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Contingencies during the ecological restoration of semi-natural grasslands

and their effects on plant species, functional traits and genetic diversity

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SUMMARY

In an attempt to halt species and habitat loss across the continent, many restoration projects have been established across Europe. However, clear scientific insight in the processes governing the success of these restoration projects is currently limited, as traditional ecological restoration research mainly focuses on the species level and the effects of site-level conditions on assembly outcome. In this study, we evaluated several underexplored aspects of community assembly following restoration within several restored calcareous grasslands in the Viroin valley in southern Belgium. In an attempt to contribute to a better understanding of how different processes shape species colonization and community assembly following restoration, we examined the effects of the landscape configuration and contingencies on community assembly at different levels of diversity organization. More specifically, we looked at the species and functional trait level assembly of the above ground vegetation and the soil seed bank, on the one hand, and the population gene level during colonization of the long-lived grassland species, *Origanum vulgare*, on the other.

Progressing assembly of the above ground vegetation was found to consist of a sequential replacement of generalist species with specialist species, which was reflected by a directional assembly at the functional trait level. Landscape configuration significantly affected this assembly, as grassland isolation slowed down assembly at both the species and the trait level. More interestingly, spatial isolation was found to act as a trait filter, independent of assembly age. We found a proportionally higher occurrence of species with light seeds and a high seed attachment potential in more isolated restoration patches, which could indicate that dispersal is likely more limited in isolated grasslands.

Furthermore, we compared differentiation among these restored grasslands based on the species and functional trait composition. These analyses showed that trait similarity among grasslands clearly increased with the amount of time since restoration, indicating trait convergence through time. At the species level, we found no evidence of convergence through time, with even a trend towards divergence. These results support the idea that only limited niches occur, which are only filled by species that have the appropriate functional traits, resulting in clear deterministic assembly at the trait level. Species identity, on the contrary, has no role in this niche filling. The first appropriate species to reach a restoration site will be the ones that get established, resulting in divergence of the species composition among restored grasslands.

When comparing the genetic diversity of recent populations and old, putative source populations of *Origanum vulgare*, we did not observe decreased genetic diversity in recent

populations, nor inflated genetic differentiation among them. Nevertheless, a significantly higher inbreeding coefficient was observed in recent populations, although this was not associated with negative effects on two measured proxies related to reproductive success. Our analyses indicated that colonization occurred from several source populations, with sufficient gene flow overcoming any large genetic founder effects, which likely increased the overall metapopulation viability of *O. vulgare*. Gene flow was nonetheless affected by the spatial configuration of the grasslands as gene flow into the recent populations mainly originated from nearby source populations.

Comparing the soil seed bank composition of restored and ancient grassland, we observed that the species richness decreased through time. This was reflected at the trait level by a replacement of traits associated with generalist therophytes by traits typical for chamaephytes and grassland specialists. While species differentiation remained relatively constant, trait differentiation was observed to decrease through time. Only the species composition of ancient grasslands was affected by spatial isolation. The seed bank composition of ancient grasslands was furthermore observed to be a nested subset of that of young grasslands. These results suggest that community disassembly occurs in the seed bank. This implicates that directly following restoration, a large and diverse seed bank is formed, followed by a gradual net loss of species. Although theory predicts this species loss to be driven by seed persistence traits, we found that this was not the case in our system, but that species loss was likely governed by functional changes in the above ground community. This disassembly process results in one deterministic end state at the trait level, but not at the species level.

Our results suggest that several parallels in assembly patterns exist among the different organizational levels of diversity, most notably among both the species and functional trait level of the above ground community and the soil seed bank. Nevertheless, clear differences among the different organizational levels also remain, illustrating the importance of a multi-level approach to gain in-depth insight in community assembly following restoration. More specifically, restoration monitoring should evaluate the genetic viability of colonizing species in parallel with community assembly since colonization itself is not a guarantee for successful establishment. The soil seed bank can furthermore significantly affect above ground assembly and should for this reason be taken into account. Finally, we observed that the spatial configuration of the study area and priority effects significantly affect assembly patterns, and should therefore be included when designing restoration projects.

SAMENVATTING

In een poging het verlies aan soorten en habitats in Europa tegen te gaan, werden in de laatste jaren ontelbare restauratieprojecten opgezet. Het wetenschappelijk inzicht in de processen die het succes van deze restauratieprojecten verzekeren, is momenteel echter nog beperkt. Traditioneel ecologisch restauratieonderzoek richt zich immers voornamelijk op het soortniveau en op de effecten van lokale omstandigheden op gemeenschapsformatie (E: *community assembly*). In deze studie bekeken we verschillende miskende aspecten van gemeenschapsformatie na restauratie, gebruik makend van de gerestaureerde kalkgraslanden in de Viroinvallei (Zuid-België). We onderzochten meer bepaald de effecten van enerzijds de landschapsconfiguratie en anderzijds toevalligheden (E: *contingencies*) op de gemeenschapsformatie op verschillende diversiteitniveaus. Op deze manier tracht_ ten we bij te dragen tot een grondiger inzicht in de processen die kolonisatie en gemeenschapsformatie na restauratie vormen. Meer specifiek bestudeerden we gemeenschapsformatie op het soort- en functionele niveau (E: *functional trait*) van zowel de bovengrondse vegetatie als de zaadbank, aangevuld met de evaluatie van de kolonisatie van de langlevende graslandsoort *Origanum vulgare* op het populatiegenetisch niveau.

Generalisten werden geleidelijk vervangen door specialisten tijdens vorderende formatie in de bovengrondse vegetatie. Dit werd vertaald in een directionele gemeenschaps-formatie op het functionele niveau. De formatie werd bovendien significant beïnvloed door de landschapsconfiguratie, meer bepaald door de ruimtelijke isolatie, wat zich vertaalde in een vertraagde formatie op zowel het soort- als het functionele niveau. Isolatie trad bovendien op als een filter voor de functionele samenstelling van de gemeenschap, onafhankelijk van de formatieleeftijd. Zo observeerden we een proportioneel hogere aanwezigheid van soorten met lichte zaden en zaden met een hoog potentieel voor aanhechting aan schapenwol en rundervacht in geïsoleerde graslandfragmenten, wat op zijn beurt op een sterk gelimiteerde kolonisatie in geïsoleerde graslanden wijst.

Vervolgens vergeleken we de differentiatie in soorten- en functionele samenstelling tussen de gerestaureerde graslanden. Deze analyses toonden aan dat de functionele similariteit tussen graslanden toeneemt met de tijd, wat op zijn beurt tot functionele convergentie leidt. Op het soortniveau werd er echter geen aanwijzing van convergentie in de tijd gevonden, met zelfs een trend naar divergentie. Deze resultaten ondersteunen het idee dat slechts een gelimiteerd aantal niches aanwezig zijn in een gemeenschap, die enkel door soorten met de gepaste functionele kenmerken kunnen ingevuld worden. Dit resulteert in een duidelijk deterministische formatie op het functionele niveau. Soortidentiteit daarentegen, heeft geen rol in

de invulling van de niches. De eerste geschikte soort die de restauratiesite koloniseert, heeft de meeste kans om er zich te vestigen, wat resulteert in een divergentie van de soorten-samenstelling tussen de gerestaureerde graslanden.

Voor *Origanum vulgare* observeerden we geen lagere genetische diversiteit in recente populaties in vergelijking met oude gevestigde populaties, noch een verhoogde genetische differentiatie tussen deze populaties. We observeerden desalniettemin een significant hogere inteeltcoëfficiënt binnen recente populaties, al was dit niet geassocieerd met negatieve effecten op twee opgemeten proxies voor reproductief success. Uit onze analyses blijkt bovendien dat kolonisatie vanuit verscheidene bronpopulaties plaatsvond, waarbij sterke stichtereffecten (E: *founder effects*) door een voldoende hoge genmigratie (E: *gene flow*) werden voorkomen. Deze nieuwe populaties leiden op deze manier tot een verhoogde metapopulatie-leefbaarheid van *O. vulgare*. Genmigratie werd evenwel beïnvloed door de landschapsconfiguratie van de graslanden, waarbij recente populaties voornamelijk genmigratie ondergingen vanuit nabijgelegen, oude populaties.

De vergelijking tussen de zaadbanksamenstelling van gerestaureerde en oude graslanden toonde aan dat de soortenrijkdom van de zaadbank daalt met de tijd. Dit vertaalde zich op het functionele niveau door een vervanging van functionele kenmerken, geassocieerd met generalistische therofyten, door functionele kenmerken, typisch voor chamaefyten en grasland specialisten. Hoewel de similariteit in zaadbanksoortensamenstelling tussen graslanden relatief constant bleef, stelden we een toename in de functionele similariteit doorheen de tijd vast. De zaadbanksoortensamenstelling van oude graslanden werd als enige leeftijdsgroep beïnvloed door de ruimtelijke isolatie en bleek bovendien een genest onderdeel te zijn van de zaadbanksoortensamenstelling van jonge graslanden. Deze resultaten suggereren dat gemeenschapsdeformatie (E: *community disassembly*) optreedt in de zaadbank. Dit impliceert dat er onmiddellijk na restauratie een grote en diverse zaadbank wordt gevormd, gevolgd door een gradueel nettoverlies aan soorten. Dit soortverlies werd, verrassend genoeg, niet gedreven door zaadpersistentiekenmerken, maar door functionele veranderingen in de bovengrondse gemeenschap. Op het functionele niveau resulteerde dit deformatieproces vervolgens in één deterministische uitkomst. Op het soortniveau bleven echter meerdere uitkomsten mogelijk.

Onze resultaten suggereren dat er tussen de verschillende diversiteitniveaus versc heidene parallellen bestaan in de gemeenschapsformatiepatronen, meer bepaald tussen zowel het soort- als functionele kenmerkniveau van de bovengrondse gemeenschap en de zaad-

bank. Er bestaan echter ook duidelijke verschillen tussen de verschillende diversiteit-niveaus, wat het belang van een aanpak op meerdere niveaus aantoont indien men een volledig inzicht in gemeenschapsformatie na restauratie wil bekomen. Meer specifiek zou restauratie-opvolging meer aandacht moeten besteden aan de genetische leefbaarheid van koloniserende soorten in parallel met gemeenschapsformatie, aangezien kolonisatie op zich geen garantie is voor een succesvolle vestiging. Ook de zaadbank zou in rekening moeten gebracht worden, aangezien deze de bovengrondse formatie significant kan beïnvloeden. Ten slotte zou de ruimtelijke configuratie van het studiegebied en prioriteitseffecten in rekening moeten gebracht worden tijdens het ontwerp van restauratieprojecten aangezien beide processen de gemeenschapsformatiepatronen in onze studie significant beïnvloedden.

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LIST OF ABBREVIATIONS

NMDS: non-metric multi-dimensional scaling
LMM: linear mixed models
REML: restricted maximum likelihood (linear mixed models)
S: species richness
D: Simpson's index of diversity
E: (Simpson) evenness
Spec.: specialist species
Gen.: generalist species
A.P.: attachment potential, the percentage of seeds still attached to a mechanically shaken animal coat after one hour (2400 swings) (Römermann et al. 2005).
$\bar{t}$: community-weighted trait mean (similar to CWM)
CWM: community-weighted trait mean (similar to $\bar{t}$)
σ : community-weighted trait standard deviations
D_E: Euclidean distance
D_{MCE}: mean censored Euclidean distance
D_{BC}: Bray-Curtis distance
φ : probability that two colonizing individuals originate from the same source population
φ_p . mean φ for one population
LI: seed bank longevity index
A: mean number of alleles per population
H_O: observed heterozygosity
H_E: expected heterozygosity
F_{IS}: inbreeding coefficient
F_{ST}: in chapter 4: pairwise genetic differentiation among populations based on Wright`s F-statistics; in chapter 5: community drift, community analogue of the fixation index in population genetics
LD: linkage disequilibrium
TPM: two-phase model of mutation
GLM: general linear model
%F: percentage of female plants in the population
G'_{ST}: pairwise genetic differentiation among populations as defined by Nei (1973) standardized by the maximum value it can obtain ($G_{ST(max)}$) (Hedrick 2005).
Jost's D: pairwise genetic differentiation among populations calculated based on the effective number of alleles instead of heterozygosity (Jost 2008).
SD: standard deviation
β_{RC} = Raup-Crick β-diversity
D_{BCS} = Bray-Curtis dissimilarity in species composition
D_{BCT} = Bray-Curtis dissimilarity in trait composition

CHAPTER 1.

GENERAL INTRODUCTION

1.1 GRASSLAND RESTORATION ECOLOGY

Large-scale habitat destruction and fragmentation have resulted in biodiversity loss and increased species rarity across Europe (e.g. Foley *et al.* 2005; Fischer & Lindenmayer 2007). The severity of this fragmentation has advanced to such an extent that proper biodiversity conservation can only be accomplished through habitat restoration, which focuses on the enlargement and defragmentation of the remaining habitats (Rey Benayas *et al.* 2009). For restoration to succeed, a consistent guiding framework is required. When outlining restoration goals, it was traditionally assumed that after re-establishment of appropriate site-level conditions, a community would assemble following a single predictable pathway towards a fixed target state through spontaneous colonization of several target species (Matthews & Endress 2010). However, several failed restoration projects have led to the realization that suitable site-level conditions will not automatically guarantee successful community restoration (Young *et al.* 2005). Increasing evidence demonstrates that plant assembly is also influenced by landscape characteristics and historical factors at the species level, complicating clear predictions of assembly outcome (Young *et al.* 2005; Bischoff *et al.* 2009; Brudvig 2011). Looking at functional trait patterns in community ecology can, however, offer a solution to this problem. Unlike species patterns, assembly at the functional trait level is expected to be more deterministic and independent of the regional species pool and historical and spatial contingencies, allowing the formulation of broadly applicable, general restoration guidelines (Kahmen & Poschlod 2004; Fukami *et al.* 2005; Matthews & Spyreas 2010; Petermann *et al.* 2010). Although promising, many aspects of this community assembly framework following restoration have been little explored so far (cf. Brudvig 2011).

Recent restoration projects were initiated on the ecologically valuable calcareous grasslands of southern Belgium, which led to the creation of a large mosaic of grassland patches of different restoration age. These offer an appropriate system to thoroughly evaluate community assembly following restoration at several levels of community organization. Hence, this PhD thesis aims at contributing to a better understanding of how different processes influence species colonization and community assembly following grassland restoration. In this chapter, we will first describe the composition, origin and degradation of calcareous grassland communities in detail, followed by a theoretical overview of all possible drivers of species colonization and community assembly after restoration. To conclude, an overview of the different research questions addressed in this PhD thesis is presented.

1.2 CALCAREOUS GRASSLANDS

1.2.1 DEFINITION, TYPOLOGY AND DISTRIBUTION

Calcareous grasslands are relatively dry ecosystems occurring on well-drained, infertile, alkaline to neutral substrates associated with calcareous rock, chalk or lime-rich loess (WallisDeVries *et al.* 2002; Calaciura & Spinelli 2008; Silva *et al.* 2008). These communities are characterized by a wide variety of heliophilous herbs and grasses adapted to drought stress and low (macro-) nutrient levels, with the presence of numerous calcicolous species (Calaciura & Spinelli 2008). Compared to most other European grassland types, these communities are typically species rich, containing many small hemicryptophytes, geophytes and therophytes, which form an open sward (Dutoit & Alard 1996).

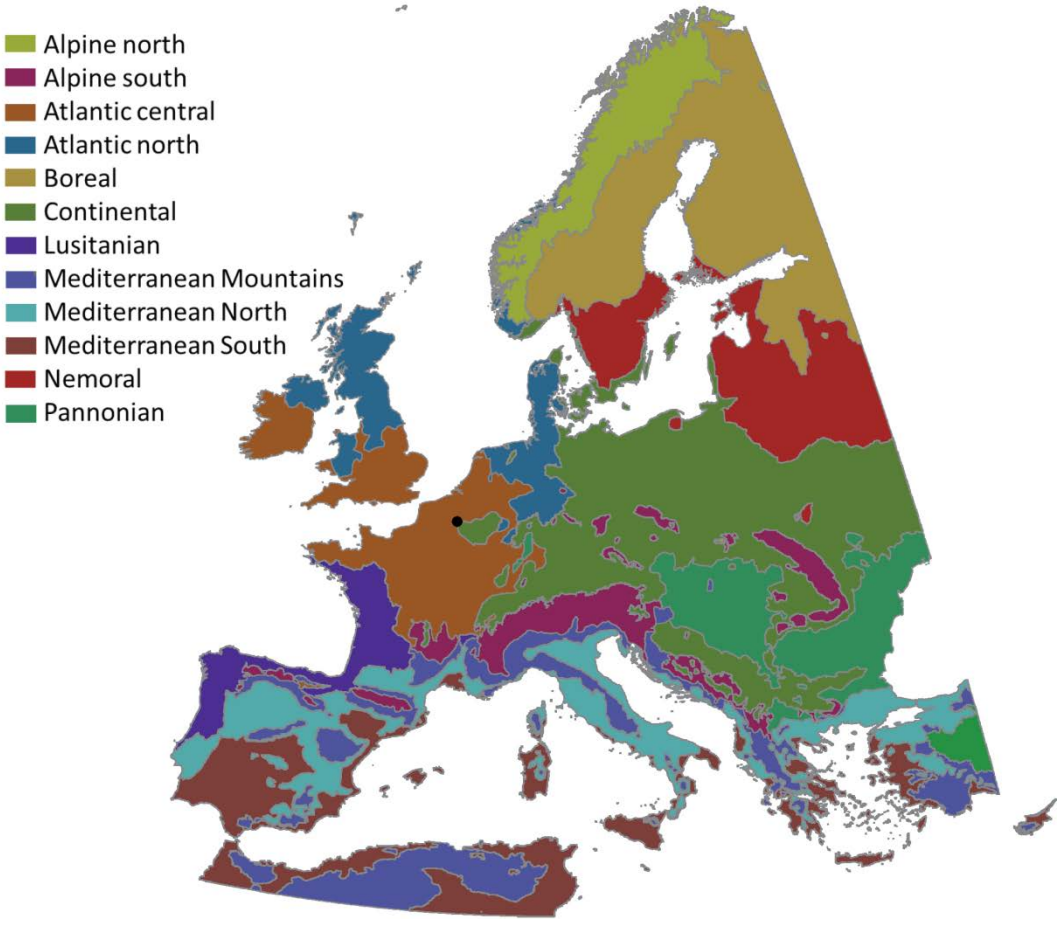
Calcareous grasslands typically occur from the lowland to the mountains in north-west and central Europe (the Atlantic, continental and sub-Mediterranean regions, Fig. 1.1) (Royer 1991). To the east, these grasslands are gradually replaced by dry steppe vegetation, while in the south, they give way to more open thermophilous Mediterranean vegetation (garrigue, maquis). As a result, the species composition of these grasslands varies considerably across Europe, with many sub-Mediterranean species in the western Atlantic and sub-Mediterranean region, and a high percentage of steppe species in the drier, eastern continental region (Willems 1982; Butaye *et al.* 2005b; Illyés *et al.* 2007).

Although highly variable in species composition, all of these grasslands can be assigned to the *Festuco-Brometea* class (Royer 1991). More specifically, all calcareous grasslands belong to two orders within this phytosociological class: *Festucetalia valesiacae* and *Brometalia erecti*/*Festuco-Brometalia* (Ellenberg 1988). The *Festucetalia valesiacae* order consists of secondary (semi-natural) grasslands containing several

Figure 1.1. Environmental zones of Europe following Metzger *et al.* (2005); visualizing the Atlantic (north and central), sub-Mediterranean (Lusitanian) and continental regions. Note that the true European steppes are confined to the Pannonian region. The location of our study area (Viroin valley) is indicated by the black dot. Figure adapted from Schmeller *et al.* (2012).

primary steppe species such as *Adonis vernalis*, *Crambe tatarica* and *Iris variegata*. This vegetation is confined to the (sub-) continental parts of Central and Eastern Europe. This order is subdivided in two alliances. One of these, the *Festucion valesiacae*, typically occurs on very dry soils and forms the transition between the true eastern steppe vegetation and more Central European calcareous grasslands. This alliance is dominated by several filiform *Festuca* and *Stipa* grasses. The second alliance, *Cirsio-Brachypodion*, occurs on deeper, moister soils and is dominated by *Brachypodium pinnatum* and *Bromus erectus*, accompanied by several continental grassland species (Ellenberg 1988).

The *Brometalia erecti* or *Festuco-Brometalia* order in turn, is confined to the Atlantic and sub-Mediterranean regions in Western Europe and parts of Central Europe (Dutoit & Alard 1996). This order also consists of two alliances, *Xerobromion*



and *Mesobromion* (Calaciura & Spinelli 2008). The *Xerobromion* grasslands comprise xeric, primary grassland communities confined to small patches of extremely dry sites on thin soils (< 5 cm), often on south-facing slopes (Royer 1991). These grasslands contain many heliophylic, sub-Mediterranean species, e.g. *Fumana procumbens* and *Hippocrepis comosa*. *Xerobromion* grassland are permanent communities, located on sites unsuitable for shrub growth, because of rocky soils, frequent fires or soil erosion (Calaciura & Spinelli 2008). Most European calcareous grasslands, however, are *Mesobromion* communities, which develop on deeper soils, leading to relatively moister conditions. These grasslands are often dominated by *Brachipodium pinnatum* and/or *Bromus erectus*, accompanied by several typically sub-Atlantic species and are known for the occurrence of numerous orchid species (Calaciura & Spinelli 2008). The grasslands studied in this thesis are mostly part of the *Mesobromion* alliance, as discussed later in this chapter (Butaye *et al.* 2005b). Please note that nomenclature for vascular plants in this thesis follows *The Plant List* (2010) Version 1, published online (http://www.theplantlist.org).

1.2.2 ORIGIN AND HISTORICAL LAND USE

Festuco-Brometea grasslands, with the exception of *Xerobromion* grasslands, are predominantly of anthropogenic origin, replacing several types of calcicolous climax forest (Royer 1991; Dutoit & Alard 1996). Although it is assumed that small patches of suitable habitat for calcareous grasslands naturally occurred after the last glaciations ('steppenheides', Gradmann 1950), most of the current calcareous grasslands can be interpreted as plagioclimax vegetation of a more recent origin (Dutoit & Alard 1996; Mortimer *et al.* 1998). During the neolithic period, primeval forests around small settlements were subjected to cutting and grazing, leading to the creation of common pastures (Pot 1996). These pastures slowly developed into calcareous grasslands on lime-rich bedrocks across Europe (Poschlod & WallisDeVries 2002). It was not until the Roman occupation that these grasslands experienced a first period of considerable expansion, as observed from palynological and macrofossil data (Pott 1996; Poschlod & WallisDeVries 2002). This expansion was largely caused by the introduction of hay-making by mowing. The accompanying expansion of the alternate husbandry system or the alternate arable field-pasture farming system from the Bronze Age to

the early Middle Ages, followed by the three-field-rotation system until the nineteenth century, further boosted the assembly and expansion of these grasslands (Poschlod & WallisDeVries 2002). These land-use practices were characterized by a rotation scheme which included an abandoned field phase.

Table 1.1. First appearance (x) of plant species occurring in calcareous grasslands in the Lower Rhine Valley (adapted from Poschlod & WallisDeVries 2002, after Knörzer 1996). Species occurring in our study system (Viroin valley, see further) are in bold. Nomenclature for vascular plants follows The Plant List (2010) Version 1. Published online (http://www.theplantlist.org).

	Neolithic period	Bronze Age	Iron Age	Roman Empire	Middle Ages
Number of sites	66	11	?	>50	>80
Euphorbia cyparissias	x	x	x	x	x
Potentilla neumanniana	x	x	x	x	x
Scabiosa columbaria	x	x	x	x	x
Silene vulgaris	x	x	x	x	x
Ajuga genevensis	x	x	x	x	x
Campanula trachelium	x	x	x	x	x
Stachys recta	x	x	x	x	x
Pimpinella saxifraga		x	x	x	x
Carex caryophyllea			x	x	x
Medicago lupulina			x	x	x
Plantago media			x	x	x
Campanula rapunculus				x	x
Centaurea scabiosa				x	x
Euphorbia seguieriana				x	x
Hippocrepis comosa				x	x
Peucedanum officinale				x	x
Primula veris				x	x
Salvia pratensis				x	x
Sanguisorba minor				x	x

It has been argued that these abandoned fields allowed the migration of many species that originated in the steppes and sub-Mediterranean region across Europe's calcareous grasslands (WallisDeVries *et al.* 2002; Pärtel *et al.* 2005). These bare soils were necessary for successful establishment of new species, since colonization by germination in fully developed calcareous grasslands is limited (Poschlod & WallisDeVries 2002). The largest phase of grassland expansion and development occurred from the fifteenth until the eighteenth century, due to the establishment of large flocks of domestic sheep, leading to transhuman shepherding and hay-making (Poschlod & WallisDeVries 2002). Since sheep are known to be good vectors of plant seeds through both endo- and epizoochory, their migration resulted in the dispersal of many species across hundreds of kilometers, which led to an increased diversity of Europe's calcareous grasslands (Willerding & Poschlod 2002; Couvreur *et al.* 2004; Adriaens *et al.* 2007). An illustration of the gradual increase in calcareous grassland area and species richness in Europe from the Neolithic period onward is presented in Table 1.1 for the lower Rhine valley (Germany).

1.2.3 RECENT DECREASE OF CALCAREOUS GRASSLAND AREA

Calcareous grasslands experienced a sharp decline in number and size from the end of the nineteenth century until the twentieth century, leading to losses exceeding 80% of the total grassland area in many European countries (Cousins 2001; Lindborg & Eriksson 2004; Adriaens 2008; Johansson *et al.* 2008). Even during the last three decades, the total grassland area in the European Union has continued to decrease by an average of 12% (Calaciura & Spinelli 2008). This decline was largely caused by the abandonment of tradition management, fueled by socio-economic changes (Muller *et al.* 1998; Poschlod & WallisDeVries 2002). Due to the introduction of mineral fertilizers, hay-making on calcareous grasslands was no longer considered economically viable. This resulted in the large scale abandonment of traditionally hayed calcareous grasslands in favour of more productive, fertile grasslands. Moreover, traditional shepherding and transhumance disappeared due to the import of cheap sheep wool, increased duties on sheep export for slaughter, and the difficult social aspects of shepherding (Poschlod & WallisDeVries 2002). This led to a reduction in the number of sheep and a transition to stationary flocks grazing more productive paddocks, which in turn led to the abandonment

of traditionally grazed calcareous grasslands. Many of these abandoned grasslands were subsequently afforested with drought resistant timber species (mostly *Pinus nigra* subsp. *austriaca*) (Adriaens 2008; Calaciura & Spinelli 2008). Village expansion, open calcareous rock mining, and increased eutrophication by run-off and aerial deposition contributed to a further decline and degradation of the remaining calcareous grassland area (Calaciura & Spinelli 2008).

The remaining fragments of abandoned calcareous grassland that escaped land use conversion were subjected to natural succession, resulting in a dramatic change in floristic composition. In the first stages, after 10-15 years of abandonment, these degraded grasslands become dominated by one or a few tall grass species (often *Brachypodium pinnatum* or *Bromus erectus*) and later on, after 15-20 years by shrubs (*Buxus sempervirens, Crataegus spp., Prunus spinosa and Rosa spp.* among others) (Willems 2001; Calaciura & Spinelli 2008). The increased litter accumulation and reduced light penetration caused by these species result in the loss of many subordinate annual and heliophilic specialist species through competition and arrested seedling establishment (Bobbink & Willems 1987). The resulting species' impoverished scrub gradually changes towards a calcicolous forest after about 30 years. The accompanied increase of forest and shrub species goes at the expense of many typical grassland species (Willems 2001). The pace of this succession, however, is strongly dependent upon the abiotic conditions, with relatively deep and fertile soils inducing a faster loss of grasslands (Willems 2001).

Small retained grassland patches that were not subjected to succession also lost several typical calcareous grassland species (Adriaens *et al.* 2006). Since these grassland patches had become small and severely isolated, populations of many species no longer received gene flow from neighboring populations. Combined with overall small populations sizes, this led to increasing deleterious effects of genetic drift, leading to the erosion of genetic variation, increasing inbreeding effects and strong genetic differentiation (Honnay & Jacquemyn 2007). In addition, the disappearance of specialized pollinators from these small grassland patches likely decreased outcrossing rates, which further increase deleterious genetic effects, in turn leading to reduced fitness, and eventually local extinction of many populations (Butaye *et al.* 2005a). Clear genetic and fitness effects of grassland isolation have indeed been demonstrated for many calcareous grassland species in Belgium (Honnay *et al.* 2007; Jacquemyn *et al.* 2010; Meekers & Honnay 2011).

1.2.4 BIODIVERSITY HOTSPOTS

While at large spatial scales (100 m^2) the highest plant diversity is observed in tropical lowland rainforest, Wilson *et al.* (2012) demonstrated that at small spatial scales (50 m^2), the highest plant diversity is found in temperate, semi-natural oligo- and mesotrophic calcareous grasslands. Indeed, the plant species richness in these grasslands often exceeds 30-40 plant species/m^2, with records of up to 80 species/m^2 (WallisDeVries *et al*. 2002). This clearly illustrates the importance of these communities as biodiversity hotspots and bio-genetic reservoirs. Among the present species, many are representatives of vulnerable groups, such as orchids (*Orchidaceae*) and gentians (*Gentianaceae*), which is especially true for *Mesobromion* grasslands. This has led to the inclusion of many of these grasslands as important orchid sites (H6210*) in the European Union Habitats Directive (European Commission 2007). In Britain, 37 vascular plant species of the national red list occur on lowland calcareous grasslands; in Wallonia (south-Belgium), this number is even higher at 54 species, further emphasising the importance of these grasslands (Mortimer *et al*. 1998, SPW 2013).

This vast richness of vascular plants is associated with an even larger diversity of arthropods, especially butterflies (Lepidoptera) and grasshoppers (Orthoptera) (Mortimer et al. 1998; Steffan-Dewenter & Tscharntke 2002). Van Swaay (2002) demonstrated that 48% of all endemic European butterfly species occur on calcareous grasslands. Moreover, of the 71 endangered European butterfly species, 52% can be found on calcareous grassland. These grasslands also form a key habitat for several grazing mammals, such as deer and rodents, and numerous bird and reptile species (Calaciura & Spinelli 2008).

Although often overlooked, calcareous grasslands provide benefits for local communities, such as employment through traditional farming and apiculture and more recently through tourism and recreation (Calaciura & Spinelli 2008). Indeed, since these grasslands harbour a large diversity of organisms, often associated with appealing landscape assets, they can attract large numbers of tourists, which can further help develop local economies (Calaciura & Spinelli 2008).

1.3 RESTORATION OF CALCAREOUS GRASSLANDS

Large losses of calcareous grassland area, combined with the growing awareness of their ecological importance, has led to increased attention for grassland conservation in the 1970's, which resulted in the reinstitution of mowing and grazing regimes across Europe (Delescaille *et al*. 1991; Poschlod & WallisDeVries 2002). The inclusion of orchid-rich *Festuco-Brometalia* (H6210*) in the EU Habitat Directive in 1992 was also triggered by these new insights (European Commission 2007). By this time, many of the remaining grasslands had become small and isolated, creating the awareness that conservation of these grassland remnants alone would not be sufficient in ensuring a sustainable future for their communities (Butaye *et al.* 2005a). The consensus grew that large scale grasslands restoration, focusing on the enlargement and defragmentation of the remaining grasslands, was required to ensure viable metacommunities (Rey Benayas *et al.* 2009). Therefore, from the 1990's onwards, the European Union provided several funds to co-finance habitat restoration projects, such as the European Fund for Rural Development (EARDF), the European Regional Development Fund (ERDF) and the Financial Instrument for the Environment (LIFE) (Calaciura & Spinelli 2008). The LIFE program was explicitly developed as a financial instrument for the conservation and restoration of habitats included in the European Union Habitat Directive (European Commission 2013). To date, three phases of the program have been completed (LIFE I: 1992-1995, LIFE II: 1996-1999, LIFE III: 2000-2006), while the fourth program, termed LIFE+, will co-finance projects until the end of 2013. Since its foundation in 1992, LIFE has co-financed 3706 restoration and conservation projects for a total of 2.2 billion euro, of which 57 focused on the restoration of *Festuco-Brometalia* grasslands across 17 European countries (Fig. 1.2) (European Commission 2013).

Calcareous grassland restoration is predominantly performed on degraded grasslands, previously converted to forest by means of active afforestation or spontaneous succession after abandonment. Grassland restoration, as interpreted in this study, consists of an initial restoration phase usually involving tree and shrub removal or cutting, followed by reinstitution of the traditional grazing or mowing regime (consolidation phase), without the active introduction of species (Willems 2001). Whether restoration will be successful in terms of species composition and diversity will not only depend on an appropriate management strategy, but also on the ability of species to quickly establish viable populations in these new grasslands (Butaye *et al*.

2005b). Indeed, after initial restoration, target grassland species are usually assumed to spontaneously colonize new grasslands, either from the soil seed bank or from nearby grasslands. The rate and success of this colonization, however, will be dependent upon the regional species pool, the site-level conditions, the landscape context, and historical factors, which could result in a large and persistent immigration credit (Jackson & Sax 2010; Brudvig 2011; Piqueray *et al*. 2011a). These four drivers of species colonization and community assembly will be discussed in detail later in this chapter.

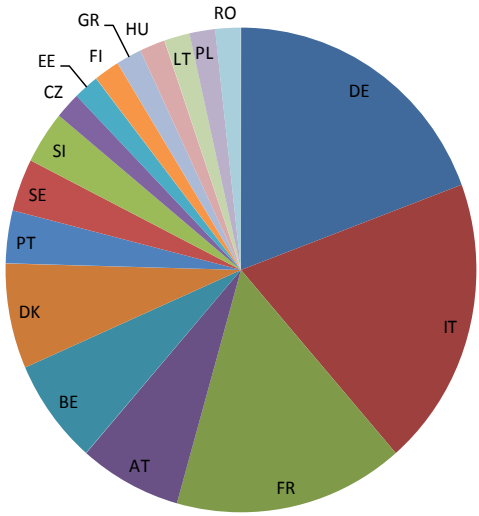


Figure 1.2. Overview of LIFE projects on the restoration of *Festuco-Brometalia* grasslands (H6210) across the European Union. Country codes correspond to international ISO 3166 codes (BE = Belgium).

1.3.1 DETERMINISTIC VS. CONTINGENT COMMUNITY ASSEMBLY

When restoration is effectuated without assisted introduction of grassland species, the grassland community formation can be interpreted as a form of secondary succession or community (re)assembly. During this process, the grassland community will assemble through gradual, spontaneous colonization of different species, finally resulting in a mature grassland community. According to this view, we can interpret the mature calcareous grassland (the restoration target) as a plagioclimax vegetation, maintained through regular grazing or mowing (Mortimer *et al.* 1998). Without traditional management, the progressing succession would result in forest (climax vegetation).

Assembly or succession has traditionally been interpreted as a deterministic, sequential replacement of species toward a single fixed stable state ('the climax model of succession', Clements 1916). This stable state was believed to be solely determined by the environmental (site-level) conditions and the regional species pool. All communities sharing one regional species pool and similar environmental conditions, are therefore expected to converge towards one common (plagio)climax community. The alternative view, on the other hand, acknowledges the influence of landscape characteristics and historical processes on community assembly ('contingent community assembly', Gleason 1927; Diamond 1975). This view has recently gained much attention, following increasing acknowledgment of the unpredictability of the assembly outcome after restoration (Drake 1991; Young *et al.* 2005; Bischoff *et al.* 2009). According to this view, priority effects, caused by variation in the sequence and timing of species arrival, and dispersal limitation, can cause large variations in species dominance and composition between communities, even under identical environmental conditions (Grman & Suding 2010). This can in turn lead to divergence in species dominance and composition between communities under identical environmental conditions, possibly resulting in several clearly distinct climax communities. These possible alterative end states have been described using several theoretical frameworks, such as alternative stable states (Beisner *et al.* 2003; Perry *et al.* 2003) and threshold models (Suding & Hobbs 2009). These frameworks assume that the alternative states can be transformed from one to the other by applying a minimum (threshold) change in certain environmental factors, but where a dregree in environmental change lower than the threshold value will be buffered, resulting in strong resilience of the alternative state (Suding & Hobbs 2009). The 'novel ecosystem' framework has also been used to describe the occurrence of alternative states, in which they are interpreted as persitent 'novel' states of the community or ecosystem compared to one expected reference state, caused by changes in abiotic and/or biotic conditions (Hobbs *et al*. 2009).

After many years of debate between advocates of both hypotheses of assembly (Samuels & Drake 1997, Pickett *et al.* 2009), awareness is growing that both alternatives should be considered as realistic trajectories (Young *et al.* 2001; Chase 2003; Fukami *et al.* 2005).

1.3.2 DRIVERS OF COMMUNITY ASSEMBLY

1.3.2.1 REGIONAL SPECIES POOL

The composition and size of the regional species pool will determine which species may colonize the restoration site, and should be taken into account when predicting restoration outcomes (Kirmer *et al.* 2008; Matthews *et al.* 2009; Brudvig 2011). The regional species pool can be defined as all species able to occupy a particular restoration site, if not limited by geographic or environmental constraints (Galatowitsch 2006). Nevertheless, other factors will determine which species present in the regional species pool will become part of the community pool on the restoration site (Zobel *et al.* 1998, Galatowitsch 2006). According to the dynamic environmental filter model, site-level conditions at the restoration site will act together with landscape and contingency factors as a combination of abiotic and biotic filters on the regional species pool, leading to a unique community species pool (Fig. 1.3) (Hobs & Norton 2004; Nuttle 2007; Brudvig 2011). In this model, the regional species pool is composed of two parts: an external species pool consisting of all species present in the areas surrounding the restoration site, and generally also an internal species pool, in the form of a persistent soil seed bank at the restoration site (Fig. 1.3) (Hobbs & Norton 2004).

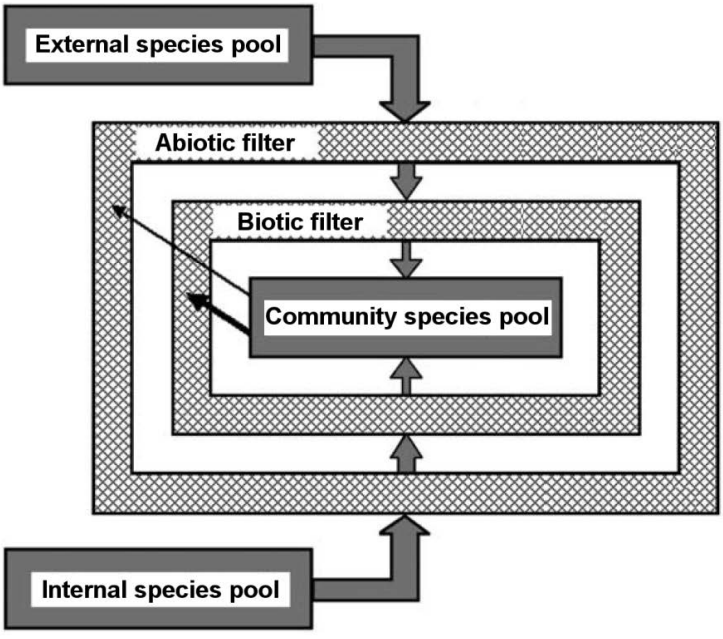
Note that according to the species pool framework of Zobel *et al.* (1998), the regional species pool will be filtered into the local species pool by the action of several biotic and abiotic filters (including an isolation filter). Accordingly, the species from the regional species pool of which both the environmental requirements can be met on the restoration site and the geographical range, includes the restoration site, make up the local species pool (Zobel *et al.* 1998; Galatowitsch 2006). The community species pool will consequently be composed of all species present in the local species pool that are

Figure 1.3. The dynamic environmental filter model. The community species pool is formed by dispersal from the surroundings (external species pool), or from the soil seed bank (internal species pool). To establish, these species must be able to pass several abiotic (climate, substrate & landscape structure) and biotic filters (competition, predation, mutualism, disturbance, order of species arrival & biological legacy). The filter mesh sizes are readjusted by internal feedback loops (actual species composition at the site), depicted as black arrows (thick arrow lines, strong effect; thin arrow lines, weak effects). Figure adapted from Hobs & Norton (2004).

able to colonize the restoration site, or in other words, that will be able to pass through the isolation filter. Note that in the dynamic environmental filter model of Hobs & Norton (2004), isolation filters are defined as abiotic filters (Fig. 1.3).

1.3.2.2 SITE-LEVEL CONDITIONS

Site-level conditions can be considered as important drivers of community assembly by creating a series of filters facilitating or inhibiting species colonization at the restoration site (Cottenie 2005; Nuttle 2007; Brudvig 2011). Site-level conditions consist of several abiotic and structural characteristics that directly interact with the community species pool, such as soil depth, fertility, acidity, slope, exposition and variation in microclimate and -topography (Young *et al.* 2005; Bischoff *et al.* 2009; Matthews *et al.* 2009). Subtle variations in site-level conditions can furthermore lead to small scale variation in species composition, which will lead to higher species diversity at the grassland scale. Periodic disturbance regimes such as burning, grazing or mowing can also be interpreted as site-level conditions (Brudvig 2011). Target species of the regional species pool can only be expected to successfully colonize the restoration site if the appropriate site-



level conditions are present (Funk *et al.* 2008; Matthews & Endress 2010). For this reason, initial steps of habitat restoration usually consist of the manipulation of the restoration site to make it suitable for the target community (Brudvig 2011). However, mere suitable site-level conditions will not guarantee successful community assembly or restoration, as has been observed in several failed restoration projects (Young *et al.* 2005). Landscape and contingency factors will also play an important role (Grman *et al*. 2013).

1.3.2.3 LANDSCAPE CONTEXT

Although often ignored in restoration studies, restoration patches are part of a larger surrounding landscape (Lindborg *et al.* 2008; Brudvig 2011). For calcareous grasslands, sources of several colonizing plant species are largely restricted to isolated grassland patches in the vicinity of the restoration patch. This is due to the highly fragmented nature of calcareous grasslands in Europe, and the absence of these species in the landscape matrix (Adriaens 2008). In this context, restoration sites can be conceived for these species as isolated patches within a hostile landscape matrix connected to mature community patches through seed and pollen flow (Holl & Crone 2004; Cook *et al.* 2005; Young *et al.* 2005). The proximity and size of these mature patches will influence the rate of assembly and the total number of species able to successfully colonize the restoration patch (Poschlod *et al.* 1998; Willems & Bik 1998; Cousins & Aggemyr 2008; Matthews & Endress 2010). The degree of hostility of the landscape matrix is nevertheless widely variable among grassland species, with several species also able to persist in certain parts of the matrix, resulting in less isolated populations when sufficient suboptimal habitat is present. But up to a certain degree, the landscape configuration acts as a dispersal filter (abiotic filter) on most species of the regional species pool by only allowing species with sufficient dispersal capacity to colonize, thus effectuating dispersal limitation for several species (Pywell *et al.* 2002; Hobbs & Norton 2004; Von Blanckenhage & Poschlod 2005). It is also worth noting that for grasslands under grazing management, migration of grazers over the different grasslands can overcome the isolation filter for several species, leading to colonization of new grasslands through both endo- and epizoochory (Fischer *et al.* 1996; Willerding & Poschlod 2002; Adriaens *et al.* 2007; Auffret *et al.* 2012). We believe that a thorough

understanding of the ecological restoration process can only be accomplished through the integration of the landscape context into the study design (Bell *et al.* 1997; Cottenie 2005; Matthews *et al.* 2009).

The landscape context can, however, affect the restoration outcome in several other ways. For instance, habitat patch size can act as an abiotic filter, by only allowing species for which the patch can sustain populations of considerable size to overcome deleterious genetic effects. However, the severity of this effect will, for each species, depend on the severity of population isolation, in turn dependent upon spatial isolation of the habitat patches, species' dispersal capacity and species' presence in the landscape matrix. Indeed, viable populations of many species will only be able to establish on restoration sites in a metapopulation context, across several grasslands connected through sufficient pollen and/or seed flow (WallisDeVries *et al.* 2002; Butaye *et al.* 2005a). Strong dispersal filters can furthermore alter the arrival order of colonizing species, which in turn can lead to priority effects (see further) (Young *et al.* 2005). As mentioned previously, the composition and quality of the surrounding non-grassland matrix landscape can also influence restoration outcome. If the matrix consists of suboptimal habitat for several grassland species or their pollen/seed vectors, restoration will be considerably easier than if the matrix consists of hostile agricultural or urban land use (Matthews *et al.* 2009; Öckinger *et al.* 2012).

Vandvik & Goldberg (2006) observed that dispersal can contribute to between 29% and 57% of the seedling diversity in perennial grasslands, but that this percentage is highly dependent upon the number of vegetation gaps suitable for germination. These gaps are in turn dependent upon the presence of grazing and mowing on these grasslands. This clearly illustrates that the positive effects of the landscape configuration on assembly rate are partly dependent upon site-level factors.

1.3.2.4 HISTORICAL FACTORS

Although traditionally overlooked in restoration ecology, the importance of historical factors on community assembly has regained much attention in recent years (Brudvig 2011). The currently most studied historical factor is the effect of variation and timing

of species arrival order during assembly (Grman and Suding 2010). These so termed 'priority effects' are responsible for a large part of the variation in species composition between different communities with similar site-level conditions, and occur when earlier arriving species affect the establishment, growth or reproduction of later arriving species by means of facilitation, competition or soil legacies (Chase 2003; Fukami *et al.* 2005; Trowbridge 2007). These priority effects can again be interpreted as a biotic filter acting on the regional species pool (Fig. 1.3) (Nuttle 2007). The occurrence of priority effects have been demonstrated both in natural systems (McCune and Allen 1985; Fastie 1995; Honnay *et al.* 2001; Collinge and Ray 2009; Grman and Suding 2010) and in mesocosm experiments (Ejrnæs *et al.* 2006; Körner *et al.* 2008). Note that this variation and timing of species arrival order is also partly governed by the landscape context, with spatial isolation filtering species based on their dispersal capacity (Foster 2001).

However, other historical factors have also been found to affect community assembly (Grman *et al*. 2013). Interannual variation in both abiotic and biotic conditions, affecting species establishment (year effects), for instance, have been hypothesized to affect assembly outcome (Bakker *et al.* 2003; Young *et al.* 2005; Cousins 2009; Vaughn & Young 2010). Historical land-use and landscape configuration (pre-assembly legacies) can also be expected to indirectly affect community assembly through direct effects on seed bank composition and quality or local species pool composition (Bakker *et al.* 1996; Stromberg & Grffin 1996; Brudvig 2011). Indeed, if a persistent seed bank is present at the restoration site, the community species pool will be largely affected by the seed bank composition, possibly altering the assembly outcome (Poschlod *et al.* 1998; Willems & Bik 1998; Von Blanckenhage & Poschlod 2005). The seed bank density and species composition can furthermore be expected to induce priority effects, by strongly influencing species arrival order.

1.3.3 FROM A SPECIES TO A FUNCTIONAL TRAIT FOCUS

Recently, plant community and ecosystem ecology have witnessed a paradigm shift from a species based approach towards a functional trait based approach (McGill *et al.* 2006; Violle *et al.* 2007). Functional traits are morphological, physiological or phenological features of species, which impact their fitness indirectly through effects

on growth, reproduction and survival (Violle *et al.* 2007). Unlike species, functional traits are believed to directly influence ecosystem functioning, properties and services, justifying the increased interest in functional trait based research (McGill *et al.* 2006; Díaz *et al.* 2007; Flynn *et al.* 2011). Using multivariate analyses, plant species can often be classified into a limited number of emergent groups, based on their functional trait attributes. This is possible since certain sets of trait attribute combinations seem to predominate in nature, reflecting the syndromes of adaptive responses and evolutionary constraints (Lavorel *et al.* 1997; Duckworth *et al.* 2000; Douma et al. 2012). Alternatively, functional traits can be used to quantify the functional diversity of a community, defined as the trait variation or dispersion in a community (Díaz *et al.* 2007). Changes in functional diversity are in turn expected to directly affect ecosystem stability and resilience (Díaz *et al.* 2007; Cadotte *et al.* 2011).

Whereas community assembly may have a strong stochastic component at the species level through the action of historical and landscape contingencies, it has been argued that at the functional trait level, community assembly can be considered to be more or less deterministic (Fox 1987; Kahmen & Poschlod 2004; Fukami *et al.* 2005; Matthews & Spyreas 2010; Petermann *et al.* 2010). This hypothesis is based on the assumption that the niches present in a community are solely defined by the environmental conditions, thus leading to similar functional groups filling up the niche space within environmentally similar communities (Matthews *et al.* 2009; Weiher *et al.* 2011). In other words, it is assumed that abiotic filters act upon the functional traits of species within the regional species pool, rather than upon the species' identity (Fukami *et al.* 2005; Cleland *et al.* 2011). Assuming the occurrence of multiple species in the regional species pool fitting any given niche can help explain the occurrence of stochastic contingent assembly at the species level (Grime 2006; de Bello *et al.* 2009).

The deterministic functional trait assembly hypothesis predicts that different communities under equal environmental conditions will converge in trait composition, but will remain differentiated, or even diverge, at the species level (Fox 1987; Kahmen and Poschlod 2004; Fukami *et al.* 2005). Indeed, many authors have reported similar trait composition in different communities under equal environmental conditions in microcosms, field experiments and natural systems (Samuels & Drake 1997; Matsui *et al.* 2002; Smith & Wilson 2002; Watkins & Wilson 2003; Fukami *et al.* 2005; de Bello *et al.* 2009; Pillar *et al.* 2009). This observed similarity in functional trait composition among communities has been termed 'community texture convergence' (Fig. 1.4).

These deterministic patterns can lead to a better insight of community assembly and dynamics, which in turn could result in clear guidelines for restoration and management (Klimkowska *et al.* 2010; Sandel *et al.* 2011; Purschke *et al*. 2013; Zhang *et al.* 2013).

Habitat (site-level) filtering is furthermore believed to result in species (phylogenetic) or, more likely, trait convergence *within* one given community (Grime 2006). Since only a limited number of niches are defined by the site-level abiotic conditions, species containing only a limited number of trait-states will be able to successfully establish in these communities, resulting in *local* texture convergence or 'trait clustering' (Cornwell *et al.* 2006). Biotic filters (competition) on the other hand, can be expected to lead to species (phylogenetic) and trait divergence or 'trait overdispersion' *within* a single community, because competition for resources will lead to competitive exclusion of species with too similar niches (Diamond 1975; Cornwell *et al*. 2006). Much research has been performed on the relative importance of trait clustering and trait overdispersion for community assembly (Watkins & Wilson 2003; De Bello *et al.* 2009; Bernard-Verdier *et al.* 2012; Raevel *et al.* 2012; de Bello et al. 2013; Purschke et al. 2013). Note that this *local* convergence/divergence within communities is not completely equivalent to convergence/ divergence *across* communities, as described above.

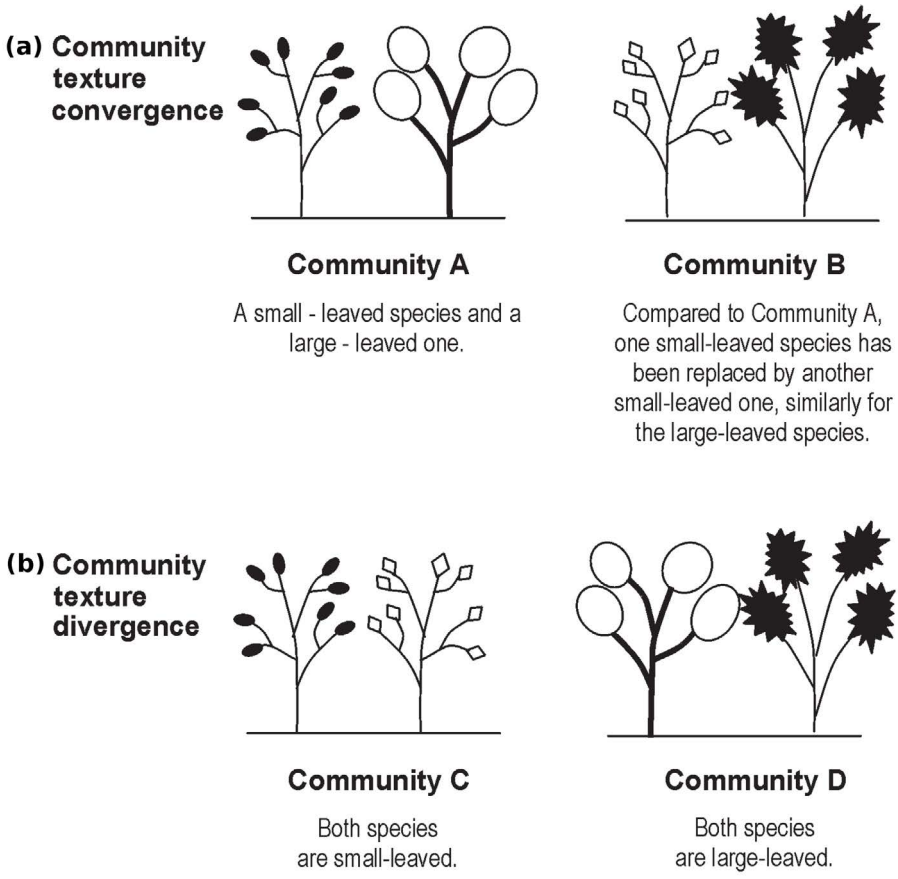


Figure 1.4. A schematic illustration of texture convergence/divergence. (a) *Community* texture convergence: two communities that differ in species composition, but have converged in leaf size (functional trait). (b) *Community* texture divergence: two communities that differ both in species composition and in leaf size (functional trait). Note that communities A and B also show *local* texture divergence ('trait overdispersion') in leaf size within the community and that communities C and D show *local* texture convergence ('trait clustering') in leaf size within the communities. Figure adapted from Matsui *et al.* (2002).

1.3.4 COLONIZATION & THE GENE LEVEL

Community assembly can only occur through successful colonization and subsequent population establishment and persistence of different plant species. The long-term viability of these newly established populations, and thus communities, can be expected to heavily depend on their genetic make-up (Lande 1988; Jamieson & Allendorf 2012). Since colonization often involves the establishment of a limited number of founding individuals, only a subsample of the genetic variability of the source populations will be present in the colonizing populations. These founder effects, or genetic bottlenecks, reduce local population genetic diversity and can result in large genetic differentiation between colonizing population (Nei *et al.* 1975; McCauley 1991). The occurrence of these founder effects is dependent upon the number of colonizing individuals and the number of source populations from which they originate (Slatkin 1977; Whitlock & McCauley 1990). In general, genetic founder effects are predicted to be strong when colonization occurs from a limited number of source populations and few colonists. Consequently, founder effects will be weak or even absent, when populations are established from multiple source populations (Slatkin 1977; Pannell & Charlesworth 1999). Notwithstanding its utmost importance for restoration ecology,

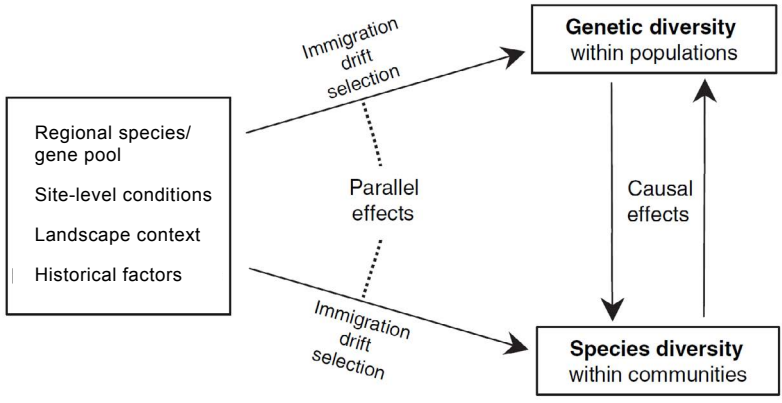


Figure 1.5. Potential connections between species diversity and genetic diversity. Note that the effect of speciation/mutation is confined within the effects of the regional species/gene pool. Immigration can be defined as dispersal at the community level and gene flow at the species level. Figure modified from Vellend & Geber (2005).

spontaneous genetic colonization patterns after habitat restoration have received relatively little attention (Montalvo *et al.* 1997; Brudvig 2011; but see Travis *et al.* 2002; Van Looy *et al.* 2009; Vandepitte *et al.* 2012).

Although not often acknowledged, many parallel effects exist between patterns governing genetic diversity within populations, and species diversity within communities (Vellend & Geber 2005; Hardy & Senterre 2007; Vellend 2010). This realisation has led to the observation of positive correlations between species richness and gene diversity in a number of studies (Vellend 2004; He *et al.* 2008; Odat *et al*. 2010; He & Lamont 2011). Nevertheless, this pattern does not seem to be generally applicable (Fady & Conord 2010; Struebig *et al*. 2011; Taberlet *et al.* 2012; Wei & Jiang 2012). Vellend (2010) hypothesized that community structure and genetic diversity within species are governed by four general, analogous processes, namely *speciation/ mutation*, *immigration*, *selection* and *drift* (Fig. 1.5). For population ecology, these four processes are indeed widely acknowledged and will therefore not be further discussed here (Lowe *et al.* 2004). At the community level, however, these concepts are relatively new. *Speciation* is interpreted as the community analogue of mutation and acts on community structure as one of the factors leading to the formation of the regional species pool. The effects of *immigration (dispersal)* have traditionally been acknowledged within community ecology, for example within the metacommunity framework and the previously discussed framework of community assembly (Leibold *et al.* 2004; Brudvig 2011; Weiher *et al.* 2011). *Selection* at the community level is interpreted as deterministic changes in community composition driven by deterministic fitness differences between individuals of different species, thus forming an umbrella term for processes as diverse as competition, niche occupancy and predation (Vellend 2010). Within community assembly this can be interpreted as the effects of site-level conditions. *Drift*, finally, can be interpreted as all stochastic changes in community composition caused by contingencies or chance, as argued by Hubbell (2001). This drift component envelops, possibly in combination with species-driven selection, assembly stochasticity and contingencies.

Extending the idea of similarity among the species and gene level to community assembly and accompanying population formation, we can predict that genetic patterns and the occurrence of founder effects during population formation are affected by the same, aforementioned drivers that affect species patterns during community assembly (Fig 1.5). The regional gene pool, partly formed through *mutation* together with other processes such as genetic recombination during sexual reproduction, will define what

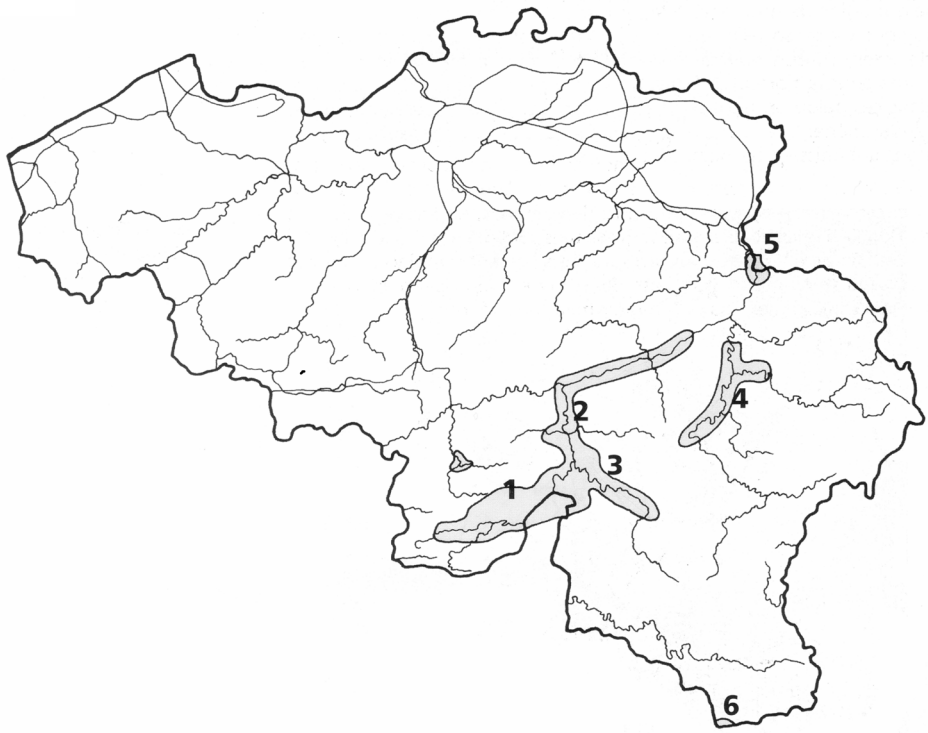
alleles will be able to occur in the local population, analogous to the effects of the regional species pool at the community level. Site-level conditions will once again act as a filter, only allowing genotypes (ecotypes) that are adapted to the local environment (*selection*), thus defining the local gene pool (Hufford & Mazer 2003). Landscape factors, such as the spatial configuration and the connectivity of restored populations and source populations, will also strongly affect colonization outcome by directly governing gene flow and colonization rates (*immigration*) (Giles & Goudet 1997; Montalvo *et al.* 1997; Austerlitz *et al.* 2000). Historical factors can be expected to manifest itself at the gene level through the introduction of locally extinct genotypes from plants originating from the persistent seed bank or effects of land-use legacies (Vellend 2004). Priority effects can also be observed when early stage genetic founder effects remain persistent, leading to very different allele frequencies of the new populations compared to surrounding old, established populations (combination of *drift* and *selection*) (Boileau *et al.* 1992).

Several causal effects can be identified between population genetic diversity and community species diversity (Fig. 1.5). Indeed, increased genetic diversity can enhance population fitness and therefore decrease extinction risk, which can lead to higher species richness at the community level compared to communities containing species with low genetic diversity, underlining the importance of large genetic diversity of target species in a conservation and restoration context. The genetic diversity of dominant species can furthermore affect community species diversity in two other ways (Vellend & Geber 2005; Gibson *et al*. 2012). First, different genotypes of the dominant species can favour different species in competition, which will result in higher species richness if the genotypes create a spatially varying selection landscape. Second, resource use efficiency or productivity of the dominant/matrix species can be increased through high genetic diversity, possibly excluding several competing species from the community, resulting in lower species richness. High species diversity can in turn reduce genetic diversity by limiting the number of genotypes of a given species able to co-exist with competitive species in the community, or the other way around, promote genetic diversity by causing diversifying selection (Vellend & Geber 2005). Alternatively, high species richness can lead to smaller population sizes of the present species, in turn resulting in lower genetic diversity.

Figure 1.6. Distribution of calcareous grasslands in Belgium (adapted from Adriaens 2008, after Decocq *et al.* 2004). 1: Viroin valley, 2: Meuse valley, 3: Lesse & Lomme region, 4. Ourthe & Aisne region, 5. Sint-Pieter mountain, 6. Gaume region.

1.4 STUDY AREA

The calcareous grasslands of Belgium are confined to six distinct areas; the Meuse valley, the Lesse & Lomme region, the Ourthe & Aisne region, the Sint-Pieter mountain and the Viroin valley (Fig. 1.6). The grasslands of the Meuse valley occur on the steep riverbanks of the river Meuse on a mixture of Devonian and Carboniferous formations. The grasslands around the Sint-Pieter mountain in the east occur on Mesozoic riverine chalk depositions, while the grasslands of the Gaume region in south Belgium occur on Jurassic calcareous rock. The three remaining areas (Viroin valley, Lesse & Lomme and Ourthe & Aisne region) are situated on calcareous outcrops of Devonian origin (Van Speybroeck 1989). This SW-NE oriented, 5 km wide, 130 km long belt of Devonian calcareous rock is locally known as the Calestienne. The Calestienne is bordered by clayer shale material to the north (Fagne-Famenne depression) and the south (the Ardennes plateau). The Calestienne grasslands all belong to the *Brometalia erecti* order and are characterized by more Atlantic communities containing several sub-Mediterranean species, which reach their northern distribution edge in



these communities (Fig. 1.1) (Butaye *et al.* 2005b; Piqueray *et al.* 2007). Detailed phytosociological research revealed that the majority of the calcareous grasslands of the Calestienne belong to the *Mesobromion* alliance. Small patches of drier grasslands, however, also occur. These grasslands are classified as true *Xerobromion* grasslands at the northern edge of the alliance's distribution by Butaye *et al.* (2005b), but are interpreted as intermediate between *Mesobromion* and *Xerobromion*, as part of the *Teucrio-Mesobromenion* sub-alliance by others (Willems 1982; Royer 1991; Piqueray 2007). A more detailed overview of the different plant communities of the Calcareous grasslands in the Calestienne region is provided by Butaye *et al.* (2005b) and Piqueray *et al.* (2007).

All grasslands studied for this PhD were confined to the Viroin valley, 75 km south of Brussels. The calcareous grasslands in this region are dispersed around the valley of the river Viroin, from the town of Couvin to the French border in Doische, occurring on stony hill outcrops (*tiennes*), ranging in altitudes from 150-250 m. The calcareous grasslands of the Viroin valley are nationally renowned for their high species richness, with 388 (25% of the national species richness) different plant species recorded on these

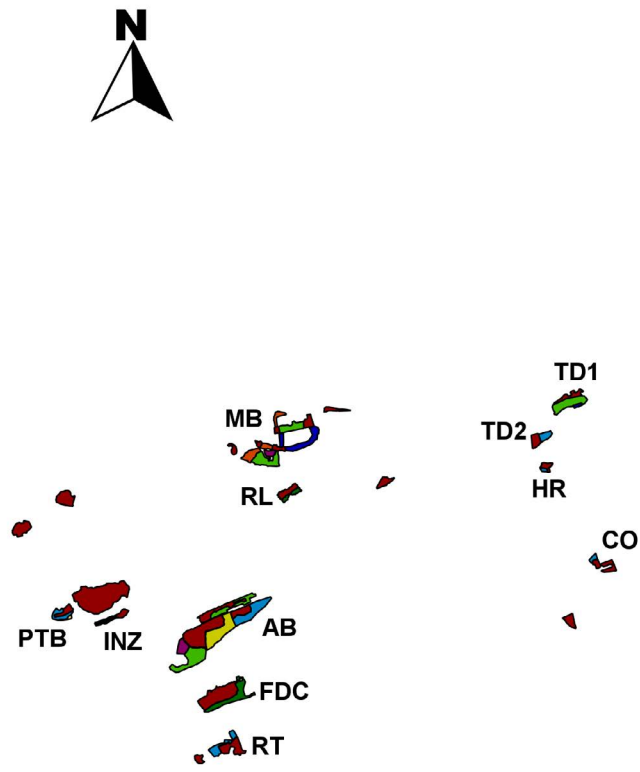


Figure 1.7. Study area in the Viroin valley, visualizing the sampled restored grasslands and the adjacent mature grasslands. Grassland codes are only given for restored grasslands and correspond to those in Table 1.2.

grasslands, containing 46 sub-national (Wallonian) red list species. In 1775, 3893.1 ha of calcareous grassland was present in the study area. This area was reduced to only 73 ha in 2002, resulting in a loss of 98.1% grassland area, which led to a large decrease in the size and number of grasslands and a strong increase in their isolation (Adriaens 2008).

Recent restoration practices have resulted in an increase in grassland area to about 200 ha (Delvingt 2006). Restoration was carried out in two phases. The first phase in 1995 led to the restoration of a small part of the Montagne aux Buis (MB) grassland, adjacent to ancient grassland (Table 1.2, Fig. 1.7). During the second phase, from 2001 to 2007, 120.58 ha of calcareous grassland was restored. This second phase was co-financed by the European LIFE project (project reference: LIFE02 NAT/B/008593, European Commission 2013). In both restoration phases, restoration practices consisted of the removal of *Pinus sylvestris* and *P. nigra* subsp. *austriaca* plantations and *Buxus sempervirens* encroachment. Following tree and shrub removal, all organic material was removed, after which spontaneous colonization of the denuded site was allowed. Soil characteristics were not directly altered, nor were plant species

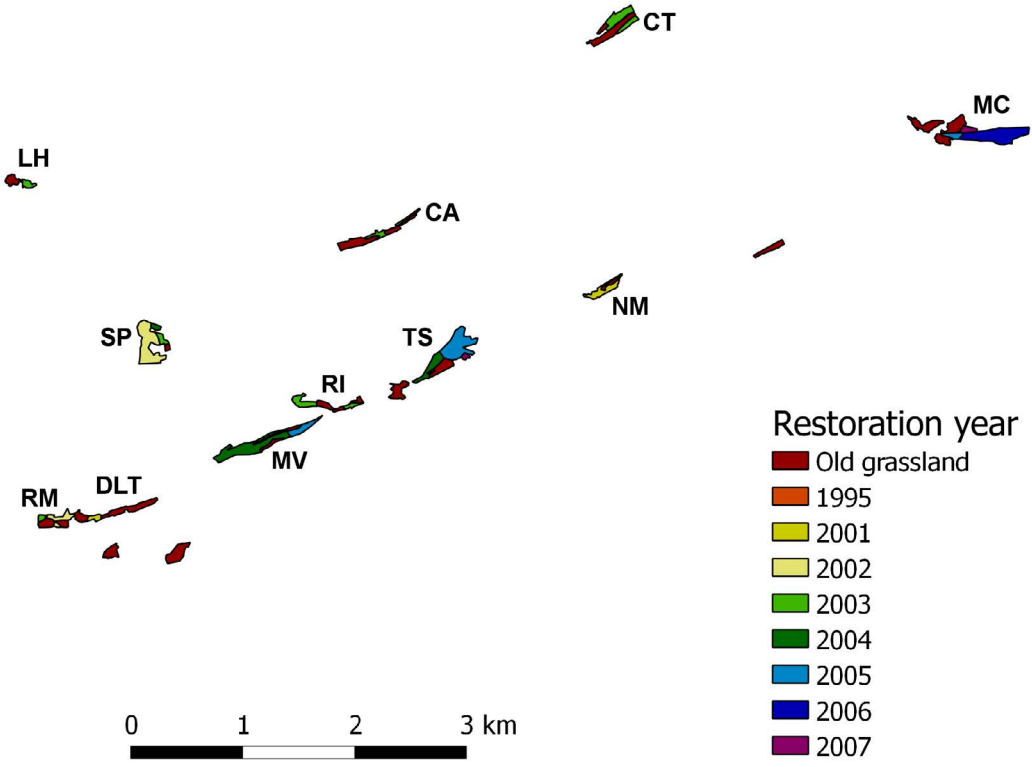


Table 1.2. Overview of the restored calcareous grasslands sampled in this study. Grassland and patch name, size and restoration year given for each grassland patch.

nr.	grassland/patch name	patch size (ha)	restoration year	nr.	grassland/patch name	patch size (ha)	restoration year
1	**Les Abannets**	**19.45**	-	**11**	**Montagne de la Carrière**	**13.34**	-
1.1	AB1	4.32	2001	11.1	MC1	0.80	2005
1.2	AB2	1.73	2003	11.2	MC2	4.92	2006
1.3	AB3	3.10	2003	12.3	MC3	0.79	2007
1.4	AB4	2.71	2005	**12**	**Mwène à Vaucelles**	**7.95**	-
1.5	AB5	1.01	2007	12.1	MV1	6.28	2004
2	**Champs d'Al Vau**	**4.01**	-	12.2	MV2	0.97	2005
2.1	CA1	1.32	2003	**13**	**Niémont**	**2.29**	-
3	**Contienau**	**1.65**	-	13.1	NM1	1.85	2001
3.1	CO1	0.35	2005	**14**	**Petit Breumont**	**1.80**	-
4	**Coupu Tienne**	**5.57**	-	14.1	PTB1	0.25	2002
4.1	CT1	2.06	2003	14.2	PTB2	1.32	2005
4.2	CT2	0.81	2003	**15**	**Rivelottes**	**2.97**	-
5	**Dessous le Transoi**	**3.59**	-	15.1	RI1	0.46	2003
5.1	DLT1	0.86	2001	15.2	RI2	1.32	2003
6	**Fondry des Chiens**	**6.93**	-	**16**	**Roche à Lomme**	**1.49**	-
6.1	FDC1	3.33	2004	16.1	RL1	0.76	2004
7	**Haute Roche**	**0.66**	-	**17**	**Roche Madoux**	**3.22**	-
7.1	HR1	0.35	2005	17.1	RM1	1.28	2002
8	**Inzevaux**	**1.13**	-	17.2	RM2	0.32	2003
8.1	INZ1	0.35	2005	**18**	**Roche Trouée**	**3.59**	-
8.2	INZ2	0.29	2006	18.1	RT1	1.31	2005
9	**Les Hurées**	**0.66**	-	**19**	**Spineu**	**8.00**	-
9.1	LH1	0.78	2003	19.1	SP1	5.24	2002
10	**Montagne-aux-Buis**	**10.35**	-	19.2	SP2	0.64	2003
10.1	MB1	1.09	1995	19.3	SP3	0.38	2004
10.2	MB2	0.45	1995	**20**	**Tienne Delvaux**	**3.47**	-
10.3	MB3	0.53	1995	20.1	TD1	1.63	2003
10.4	MB4	1.62	2003	20.3	TD3	0.29	2006
10.5	MB5	1.31	2003	**21**	**Tienne Delvaux RL**	**1.48**	-
10.6	MB6	0.41	2006	21.1	TD2	1.26	2005
10.7	MB7	1.59	2006	**22**	**Tienne Saumières**	**8.82**	-
10.8	MB8	0.44	2007	22.1	TS1	2.62	2004
				22.2	TS2	4.05	2005
				22.3	TS3	0.31	2007

introduced, resulting in overall similar mean starting conditions of the different restored grassland patches compared to the ancient grassland patches for several soil variables (N, P, K, C, Fe, pH and soil depth) (André & Vandendorpel 2004; Piqueray *et al.* 2011b). Nevertheless, small scale variation in several abiotic conditions such as soil depth and grassland slope occurs across the different restored (and ancient) grassland patches. Most restored grasslands were adjacent to ancient (mature) calcareous grasslands, from which dispersal of species could occur. Since restoration was performed over several consecutive years, adjacent to ancient grasslands, many grassland fragments consisted of a mosaic of patches of different restoration age (Table 1.2, Fig. 1.7). Throughout this thesis, uninterrupted grassland areas surrounded by other land use types will be referred to as grassland *fragments*. A grassland *patch* in turn, will be defined as an uninterrupted part of a grassland fragment of one certain (restoration) age. After initial restoration, grassland management consisted of annual grazing, identical to the management of the mature grasslands. This systematic grazing is accomplished using two sheep flocks and one goat herd, in total consisting of 300 animals (European Commission 2013). These flocks are rotated over all grassland fragments, leading to a consistent management over all grassland, nevertheless with differences in grazing time and duration among fragments. The rotation of these flocks furthermore leads to the possibility of seed dispersal through endo- and epizoochory (Adriaens *et al.* 2007).

The configuration of this grassland system, consisting of a large mosaic of grassland patches of different restoration age, enabled us to evaluate community assembly following restoration using a chronosequence approach. This approach represents a type of 'natural experiment' using a space-for-time substitution (Pickett 1989).

1.5 THESIS OBJECTIVES AND OUTLINE

Clear scientific insight in the processes governing the success of restoration projects is currently limited, as traditional ecological restoration research mainly focuses on the species level and the effects of site-level conditions on assembly outcome. As a result, our current understanding of the effects of landscape configuration and contingencies on assembly outcome on different levels of diversity organization (such as the gene

and functional trait level) is rather limited (Brudvig 2011). Using the recent restoration project on the calcareous grasslands in southern Belgium, this study aims to evaluate the process of assembly following restoration on several levels of diversity organization following initial restoration. More specifically the goals of this PhD are to:

1. Quantify how plant community assembly and richness at both the species and the functional trait level are affected by the abiotic environment and the spatial configuration of restored grassland patches.

2. Examine changes in differentiation in species and trait composition among restored grasslands with progressing community assembly.

3. Evaluate the effects of population formation through colonization on the population genetic diversity and among population differentiation in a long-lived calcareous grassland species.

4. Quantify patterns of seed bank assembly through time at the species and functional trait level and evaluate the effects of spatial isolation on seed bank patterns.

5. Compare patterns at the different studied organization levels of diversity in our study area and suggest general restoration guidelines that may be applicable to other communities.

Fig. 1.8 graphically represents the position of the different aims in relation to the different chapters, clearly illustrating the three levels of diversity organization: the gene, species and functional trait level. Restoration will lead to the colonization of several target species, which in turn leads to progressing community assembly. Both population colonization and species assembly will be affected by the regional gene/species pool, site-level conditions, the landscape context and historical factors. Functional trait assembly on the other hand will only be affected by site-level conditions and to a smaller extent to the landscape context. Community assembly was examined in this thesis through both diversity/composition and differentiation at these three organization levels. Changes in the seed bank are expected to affect assembly and colonization.

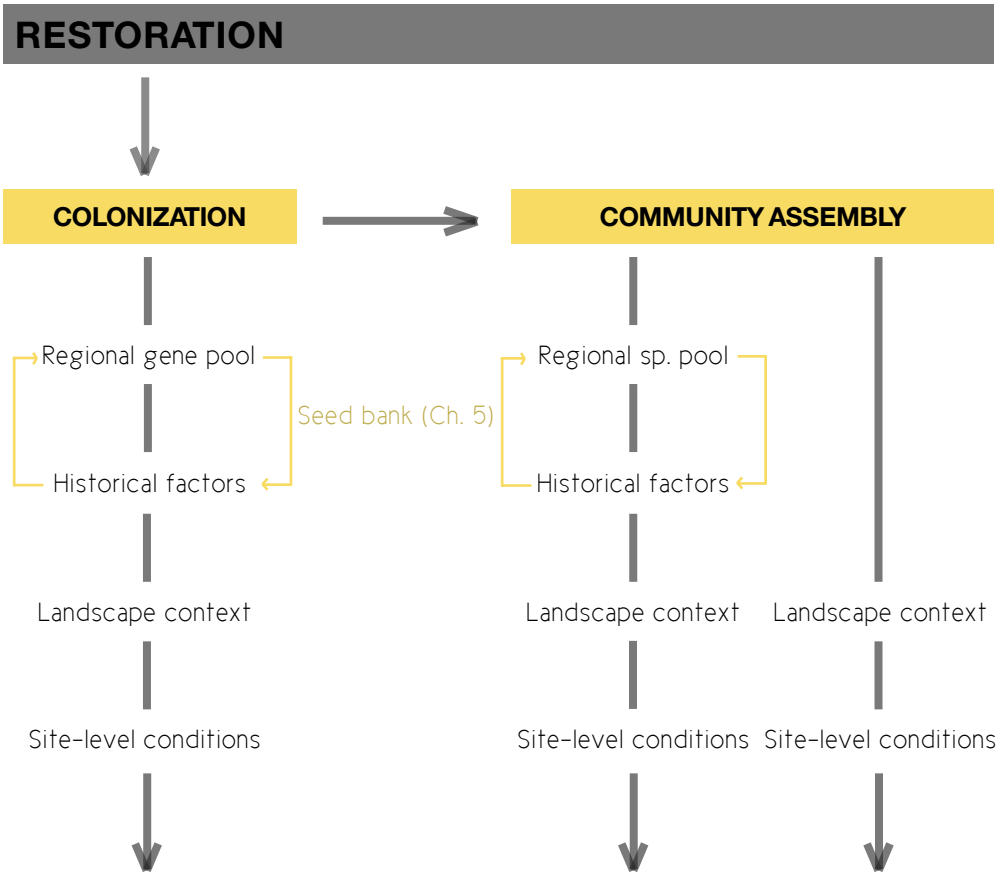


Figure 1.8. Schematic overview of the thesis indicating the position of each chapter (Ch.) into the restoration process of calcareous grasslands.

SPECIES AND FUNCTIONAL TRAIT LEVEL

CHAPTER 2

Ecological restoration research has hitherto almost exclusively focused on the species level, indicating the urgent need of more trait-based restoration studies. Additionally, the effect of the landscape configuration on restoration outcome on both the species and trait level has also hardly been investigated (Brudvig 2011). Therefore, this chapter examines how community composition changes through time at both the species and functional group level, using a chronosequence approach. Furthermore, we examine if the landscape position of the restoration sites (space) and time since restoration (time) interact in mediating community assembly, and look for plant traits that mediate recolonization.

CHAPTER 3

While community assembly at the species level is expected to be contingent on landscape and historical factors, assembly at the functional trait level has been hypothesized to be deterministic. When comparing multiple restoration sites we would expect decreasing similarity in species composition among sites with increasing age (divergence), but increasing similarity in mean trait composition (convergence) among sites. In this chapter we evaluate this hypothesis for assembly following restoration of calcareous grasslands by calculating pairwise multivariate distance (differentiation) between restoration sites and regressing them to restoration age using the chronosesquence data obtained in chapter 2.

GENE LEVEL

CHAPTER 4

The long-term stability of spontaneous community assembly following restoration is largely dependent on the genetic make-up of the founder populations of colonizing plant species. This colonization process can only be considered successful if the genetic makeup of founding populations is not eroded through founder effects and subsequent genetic drift. In this chapter we investigate the genetic effects of recent colonization of the long-lived, calcareous grassland specialist species *Origanum vulgare* on restored grassland patches in our study area. Using 10 microsatellite markers we compared the genetic diversity and differentiation of fourteen recent populations with that of thirteen old, putative source populations. Furthermore, we evaluate the effects of the spatial configuration of the populations on colonization patterns.

SEED BANK

CHAPTER 5

The importance of the soil seed bank for community dynamics is widely acknowledged through buffering of populations against disturbance and stochasticities, acting as a genetic and taxonomic reservoir for the present plant community and its mediating effects during restoration, possibly leading to priority effects. Nevertheless, little is known about how the soil seed bank is affected by above ground community assembly. In this chapter, we compare the seed bank composition of three age classes and examine how the seed bank assembles following grassland restoration at both the species and the functional trait level, taking into account the effects of spatial isolation.

SYNTHESIS

CHAPTER 6

In the last chapter, an overview of the main results of the previous chapters is given, followed by a theoretical synthesis of these findings. Furthermore, we deduce a number of restoration guidelines, applicable to unrelated grassland restoration systems. Finally, we pinpoint the shortcomings of this study and suggest several future research topics that could help further in understanding restoration and community assembly of these fascinating grassland systems.

CHAPTER 2.

ISOLATION SLOWS DOWN PLANT ASSEMBLY

Spatial isolation slows down directional plant functional group assembly in restored semi-natural grasslands

ADAPTED FROM:

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2.1 SUMMARY

Ecological restoration schemes often assume that after reinstating appropriate abiotic conditions, plant communities will assemble following a single predictable pathway towards a fixed target state. This idea has recently been challenged, with increasing evidence that plant community assembly can only be considered deterministic at the plant trait level, rather than at the species level, and that the assembly outcome is largely influenced by the spatial context of the restoration site. We surveyed 147 vegetation plots across a chronosequence of 22 restored semi-natural grassland patches to quantify the effects of spatial isolation on both plant species and plant functional trait assembly. Trait level assembly was analysed using an emergent group approach, based on 28 functional plant traits. Additionally, we examined the effects of several dispersal related plant traits on species recolonization capacities. Whereas total plant species richness of the restoration patches did not change through space or through time, progressing assembly was found to consist of a sequential replacement of generalist species with specialist species, which was reflected by a directional assembly at the plant trait level. Grassland isolation was found to slow down community assembly at both the species and the trait level without changing the general direction of assembly. This slowdown became less pronounced with increasing time since restoration. Furthermore, spatial isolation of the restoration patches was found to act as a trait filter, independent of assembly age. We found a proportionally higher occurrence of species with light seeds and a high seed attachment potential in more isolated restoration patches, suggesting that colonization is more limited in isolated grasslands. In this chapter we demonstrate that the assembly process, at both the species and the trait level, is influenced by the position of the restoration patch in the landscape. Monitoring schemes following ecological restoration should therefore include the spatial context of the system, while using both a trait based and a species based plant community analysis.

2.2 INTRODUCTION

Since habitat restoration is crucial for conserving biodiversity, a consistent framework for restoration guidance is necessary. Restoration goals are most often embodied in a reference or target plant community that guides the restoration practices (Matthews & Spyreas 2010). This approach implicitly assumes that after reestablishment of appropriate abiotic conditions, a community will assemble following a single predictable pathway towards a fixed target state (Matthews & Endress 2010), according to the classical climax concept of succession (Clements 1916). Increasing evidence, however, shows that plant assembly does not only depend on local site conditions, but that it is also influenced by landscape characteristics and historical processes (Young *et al.* 2005; Bischoff *et al.* 2009; Brudvig 2011). It has been hypothesized that historical contingency, which acts through species arrival order, can lead to priority effects (Chase 2003; Fukami *et al.* 2005; Trowbridge 2007). These effects occur when earlier arriving species affect the establishment, growth or reproduction of later arriving species by means of competition or soil legacies. These long lasting or even irreversible effects may result in a species composition that is different from the expected target community (Gleason 1927; Chase 2003).

Since priority effects are assumed to be independent of species identity, and only to depend upon arrival sequence, identifying the factors affecting species arrival are crucial (Young *et al.* 2001). Several approaches have been used to test whether propagule availability determines community assembly (Bischoff *et al.* 2009; Brudvig 2011). Seed addition experiments, for example, have demonstrated the importance of seed dispersal limitation (e.g. Turnbull *et al.* 2000; Clark *et al.* 2007; Hedberg & Kotowski 2010). Other studies have evaluated the effects of proximity to seed sources on species recolonization, reporting both strong distance effects (e.g. Bischoff *et al.* 2009; Pottier *et al.* 2009) and weak or even nonexistent distance effects (Holl & Crone 2004; Cole *et al.* 2010; Matthews & Endress 2010). These studies have shown that landscape characteristics can have large effects on the restoration outcome, indicating that a thorough understanding of the ecological restoration process can only be accomplished through integration of the landscape context of the restoration site into the study design (Bell *et al.* 1997; Matthews *et al.* 2009). Restoration sites are then conceived as isolated patches within a, for most species, predominantly hostile landscape matrix, connected to mature communities through seed and propagule flow (Holl & Crone 2004; Cook *et al.* 2005; Young *et al.* 2005).

Whereas community assembly may have a strongly stochastic component at the species level through the action of priority effects, it has been shown that at the level of trait-based functional groups, community assembly can be considered to be more or less deterministic (Fox 1987; Fukami *et al.* 2005; Petermann *et al.* 2010). This implies that unlike species based analyses, trait based analyses are more likely to elucidate general assembly patterns, which may be transferable to other restoration sites, independent of site history or the taxonomic composition of the species pool (Pywell *et al.* 2003; Kahmen & Poschlod 2004; Pottier *et al.* 2009). Although the landscape context can be expected to have little effect on the deterministic trajectory of trait assembly, it is believed that isolation influences trait assembly in a more subtle way. It has indeed been shown that a species' dispersal capacity is related to several species traits (Thomson *et al.* 2011). In this way isolation can act as a trait filter, altering the species composition of the community (cf. Clark *et al.* 2007; Lindborg *et al.* 2011).

In the present study, we investigated the restoration process in fragmented semi-natural calcareous grasslands in southern Belgium. These extremely species rich grasslands were once common across Europe and were maintained by regular grazing and cutting (WallisDeVries *et al.* 2002; Pärtel *et al.* 2005). Land-use changes during the last century have, however, led to a severe reduction of their extent (Poschlod & WallisDeVries 2002; Adriaens *et al.* 2006). To prevent total loss of these species rich communities, restoration of abandoned grasslands has become common practice (e.g. Poschlod *et al.* 1998; Butaye *et al*. 2005a). Here we try to explore a partly neglected domain of restoration ecology by combining the plant trait response to ecological restoration with an analysis of the grassland community assembly process at the landscape scale. We used plant species abundance data from 147 plots across 22 semi-natural calcareous grasslands, restored over a twelve year time span, to answer the following questions:

1. Does the landscape position of the restoration sites (space), and time since restoration (time) interact in mediating community assembly at the species level, making the assembly process contingent on the position of the restoration patch in the landscape?

2. Is community assembly at the trait level influenced by the landscape position of the restoration sites?

3. Does grassland isolation act as a trait filter, independent of restoration age, with respect to dispersal traits?

2.3 MATERIALS AND METHODS

2.3.1 SPECIES COMPOSITION SURVEY

The restored grasslands were surveyed using two 2 x 2 m plots for every restored hectare, randomly spaced over the grassland (see chapter 1.4 for a detailed description of the study area). In total, 147 plots were established in 46 restoration patches (of different restoration age, ranging from 3 to 15 years), in 22 grasslands (Table 1.2, Fig.1.7). Species occurrence and abundance (% cover) of all plants (tracheophytes) were recorded in the plots during spring and early summer of 2010. Abundance data were obtained by dividing the 2 x 2 m plots into four smaller 1 x 1 m subplots. Abundance was then estimated for each subplot using fixed abundance groups (1-2-5-10-15-20-25-30-... % cover). Total plot abundance of a species was calculated as the mean abundance across the four subplots.

2.3.2 DATA ANALYSIS

2.3.2.1 SPATIAL & ENVIRONMENTAL VARIABLES

Four different spatial isolation metrics were calculated for each plot, based on existing vegetation maps of the study area, using QGIS 1.5.0 (Quantum GIS Development Team 2010). Closest edge distance was defined as the Euclidean distance between the plot and the closest edge of the nearest mature calcareous grassland patch. The closest centroid distance was defined as the Euclidean distance between the plot and the centroid of the nearest mature calcareous grassland patch. A buffer isolation measure was defined as the total area of mature calcareous grassland present within a 1500 m radius around the plot. Finally, the Hanski isolation measure was defined as $\sum \exp(-d_j) A_j$ (Hanski 1999), where A_j is the area of the j^{th} mature fragment located at a distance d_j from the plot, for j = 1 to k, with k equal to the number of patches within a buffer of 1500 m.

In order to rule out the effects of environmental variation between grasslands that may co-vary with restoration age or isolation, we surveyed the following abiotic variables in each 2 x 2 m plot: cover (%) of bare rock and open soil, soil depth and plot inclination. These variables were averaged for each grassland patch. Using QGIS we calculated the mean elevation and isolation (as defined previously) of each grassland patch. Except for the cover of bare soil, none of these variables were significantly correlated with grassland patch age (years since restoration) (Appendix 2.1), and they are not discussed any further. Soil depth and plot inclination were found to be significantly correlated with grassland patch isolation and were included in further analyses (Appendix 2.1). We also tested for the occurrence of spatial autocorrelation for grassland patch age and isolation by calculating Moran's I in SAS 9.2 (SAS Institute Inc. 2004). Although grassland patch age showed no significant spatial autocorrelation, grassland patch isolation was found to be spatially autocorrelated (Appendix 2.1).

2.3.2.2 EMERGENT GROUP DELINEATION

28 plant traits were selected for emergent group delineation (Appendix 2.2). Traits were chosen based upon their relevance for community assembly, including the processes of dispersal, establishment and persistence (cf. Weiher *et al.* 1999). Trait values were retrieved from different sources (Fitter & Peat 1994; Thompson *et al.* 1997; Bekker *et al.* 1998; Lambinon *et al.* 1998; Klotz *et al.* 2002; Poschlod *et al.* 2003; Kleyer *et al.* 2008). Two measures of seed attachment potential, one for cattle fur and one for sheep wool, were calculated according to Römermann *et al.* (2005), using information on seed morphology following Cappers *et al.* (2006). In total, 92% of all trait values were available for all species. Emergent groups were delineated following the approach of Verheyen *et al.* (2003), using ClustanGraphics 8 (Wishart 2006). First, the similarities between species were calculated based upon the trait values. We used Gower's similarity coefficient, since it can cope with both missing data and mixed data types (binary, ordinal, nominal and ratio) (Gower 1971). The resulting similarity matrix was used for minimum variance clustering of the species into emergent groups (Ward's method, Ward 1963). The optimal number of clusters was determined using the tree validation procedure available in ClustanGraphics 8 (Wishart 2006).

2.3.2.3 COMMUNITY COMPOSITION THROUGH ORDINATION

To analyse community composition, two Non-Metric Multi-dimensional Scaling ordinations (NMDS) of Bray- Curtis dissimilarity matrices were performed; one on the arcsine square root transformed *plots x species* matrix, and one on the arcsine square root transformed *plots x emergent groups* matrix, using PC-ORD 5.33 (McCune & Mefford 1999). To avoid convergence on a suboptimal solution, we repeated the iterative NMDS algorithm 250 times. We selected a three-dimension model based on stress reduction with a mean stress value of 17.2 and 16.6, respectively, for the species and plot ordination (McCune & Mefford 1999). The *plot x emergent groups* matrix was created with emergent group abundance equal to the summed abundances of all species present in that plot, belonging to the emergent group. Correlations between the plot scores on the three NMDS axes and restoration age, spatial isolation and their interaction, were analysed using a linear mixed model (REML). Soil depth and plot inclination were added to the model as covariates. Since plots were located within the 22 grasslands, data were not independent. Therefore, grassland identity was included as a random factor, taking into account the spatial clustering of data within 22 independent groups. Semi-partial R^2_β coefficients were calculated for each covariate using the method of Edwards *et al.* (2008).

2.3.2.4 DIVERSITY METRICS

Species richness (S), Simpson diversity (D) and evenness (E) were calculated for each plot, including all species, generalist species only, and specialist species only. Simpson diversity per plot was defined as $D = 1 - \sum_{i=1}^{S} p_i^2$, with p_i the relative abundance of species i in the plot. Evenness was calculated by dividing the Simpson diversity (D) by the species richness (S) for each plot. Specialist species were defined as species confined to calcareous grasslands in Belgium (Lambinon *et al.* 1998; Van Landuyt *et al.* 2006) (Appendix 2.3). Species richness was also calculated for all derived emergent groups separately. Linear mixed models analogous to those performed on the

NMDS plot scores were performed on all diversity indices. D and E were logarithmically transformed to obtain a normal distribution. Species richness of specialist species, generalist species and the different emergent groups were divided by the total species richness of each plot to obtain the proportional richness, before analysis with REML.

2.3.2.5 TRAIT RESPONSE ANALYSIS

For all species, occurring in 10 to 90% of the plots (108 out of 247 species), the effect of restoration age and grassland isolation on their presence/absence was examined using full-factorial multivariate logistic regression models. The resulting β-coefficients of the logistic regressions for restoration age and patch isolation can be considered as a measure of the importance of these variables in driving species distribution (Dupré & Ehrlén 2002). To elucidate the mediating role of plant dispersal capacity, we evaluated the relationship between these β-coefficients and eight plant traits, using Spearman rank correlations. Plant traits used in these analyses were chosen for their relevance to the dispersal process: plant height, seed length, seed shape, seed longevity, seed mass, seed number and the earlier calculated metrics of attachment potential, one for cattle hair and one for sheep wool. Since only 44% of all species were included in the logistic regression analyses, we tested for biases in the representation of generalist/specialist species and species of the different Emergent groups using two χ^2-tests. All statistical analyses were performed in SAS 9.1 (SAS Institute Inc. 2004).

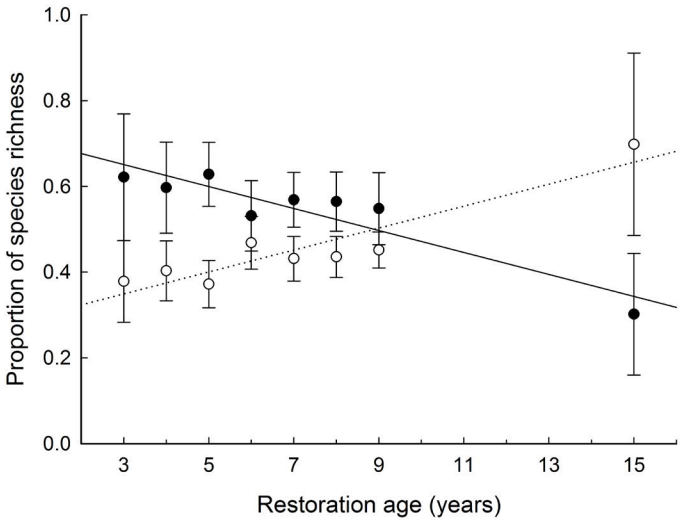


Figure 2.1. Change in proportional species richness through time after restoration for generalists (full circle, continuous line) and specialists (open circle, dotted line). Overall mean and 95% confidence intervals are presented for each restoration age class.

2.4 RESULTS

The plot scores on the first NMDS axis, for both the species composition and emergent group composition, were significantly correlated with both restoration age and grassland isolation, with plots in the oldest and least isolated communities clustering at low values of the first ordination axis for the species ordination, and with the opposite pattern apparent in the emergent group ordination graph (Table 2.1, Appendices 2.4 & 2.5). The significant interaction terms, however, indicate that differences in plot scores caused by isolation are becoming smaller with increasing grassland age (Table 2.1). Plot scores of the second NMDS axis were also significantly correlated with isolation for the emergent group ordination (Table 2.1). Since all isolation metrics were intercorrelated (Appendix 2.1), only the results for the distance to the closest edge are reported. We found no effects of restoration age or grassland isolation on the diversity indices that include all species. The proportional specialist species richness, however, increased with time since restoration and decreasing isolation, indicating independent effects of restoration age and spatial isolation. The opposite was true for the proportional generalist species richness (Table 2.1, Figs 2.1 & 2.2). Note that the proportional generalist species richness is the one-complement of the proportional specialist species richness, explaining the equal test results (Table 2.1). Specialist species evenness

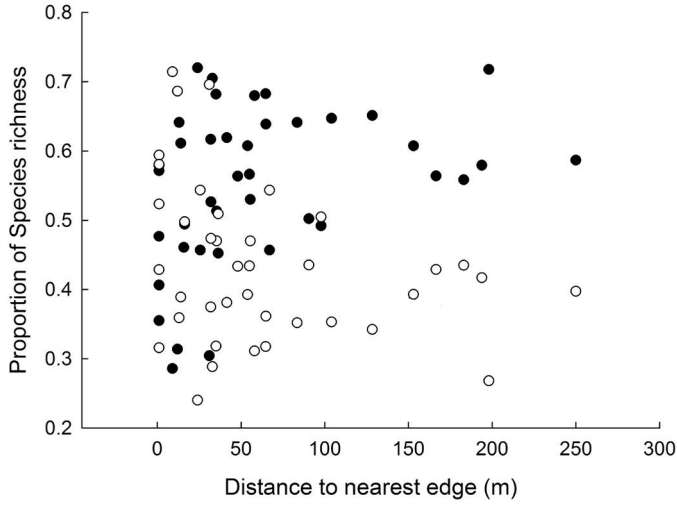


Figure 2.2. Change in the proportional species richness with increasing grassland patch isolation for generalists (full circle) and specialists (open circle). Data points are the mean richness values for each separate grassland patch.

	age			isolation			age*isolation			soil depth			inclination		
	β	F	R^2_β	β	F	R^2_β	β	F	R^2_β	β	F	R^2_β	β	F	R^2_β
NMDS axis 1 (species)	-2.38***	13.1	0.098	30.93*	5.5	0.044	0.015*	5.5	0.044	0.68**	7.9	0.062	-2.18*	4.4	0.035
NMDS axis 2 (species)	0.021	<0.01		-0.017	1.0					-0.86**	8.8	0.068	3.7**	10.5	0.079
NMDS axis 3 (species)	2.08**	10.0	0.076	0.015	0.8					-0.26	0.7		-0.043	<0.01	
NMDS axis 1 (Em. groups)	2.01**	6.9	0.054	-44.34**	8.5	0.066	0.022**	8.5	0.066	-0.48	2.9		-0.7	0.4	
NMDS axis 2 (Em. groups)	-1.06	1.2		41.19*	4.5	0.036	-0.021*	4.6	0.037	0.42	1.3		-1.61	1.4	
NMDS axis 3 (Em. groups)	0.41	0.4		0.011	0.4					0.69	5.1		0.49	0.2	
S	0.074	0.08		0.0023	0.1					-0.022	0.03		-0.022**	8.6	0.066
S spec.	0.022***	23.8	0.16	-0.00034**	7.5	0.059				-0.0063**	8.8	0.068	0.020*	6.0	0.047
S gen.	-0.022***	22.6	0.16	0.00034**	7.9	0.061				0.0053*	6.2	0.049	-0.023**	7.5	0.058
D	-0.0090	0.1		0.00019	0.09					-0.0028	0.05		-0.11*	6.4	0.050
D spec.	0.0043	0.3		-0.00012	0.4					-0.0067	3.1		-0.028*	4.4	0.035
D gen.	-0.0024	0.09		0.00031	2.0					0.00085	0.04		-0.013	0.8	
E	-0.0038	1.0		-0.00004	0.1					0.00020	0.01		-0.0047	0.5	
E spec.	-0.019**	7.5	0.059	0.000043	0.05					0.0021	0.4		-0.015	1.6	
E gen.	0.018*	5.1	0.041	-0.00009	0.2					-0.0026	0.5		0.042**	9.4	0.072
S Em. group 1†	-0.0056*	4.5	0.036	0.082**	6.9	0.055	-0.00004**	6.9	0.055	0.00026	0.08		0.0072*	6.8	0.053
S Em. group 2†	-0.0086*	4.2	0.034	0.14*	6.7	0.053	-0.00007*	6.7	0.053	0.00050	0.1		0.0062	1.3	
S Em. group 3†	-0.00099	0.9		0.0000078	0.2					0.00020	0.2		-0.0013	0.5	
S Em. group 4†	0.020***	11.6	0.088	-0.20**	7.4	0.058	0.00010**	7.4	0.058	-0.0038*	4.1	0.033	-0.018*	6.1	0.048
S Em. group 5†	-0.012***	15.0	0.11	0.00013*	4.7	0.038				-0.00012	0.01		-0.010	3.4	
S Em. group 6†	0.0021*	4.1	0.033	-0.00006*	4.4	0.035				-0.00083	1.4		0.0017	0.4	
S Em. group 7†	0.0062*	6.3	0.050	0.000040	0.7					0.0028*	6.3	0.049	0.011*	5.9	0.046

Table 2.1. Parameter estimates of the model relating NMDS axes and diversity indices to restoration age and isolation using REML. β-coefficient, test statistic and semi-partial R^2_β given for restoration age, isolation and the interaction term, soil depth and inclination (n = 147). Models with a non-significant interaction term were rerun using the main effects model. S = proportional species richness, D = logarithmic transformation of Simpson diversity, E = logarithmic transformation of evenness. † = see Table 2.2 for names and contents. Significance: *0.05 ≥ *P*-value > 0.01 **0.01 ≥ *P*-value > 0.001 ***0.001 ≥ *P*-value

showed the opposite trend regarding the time since restoration effect and decreased with progressing assembly, indicating that an increase in specialist species number paralleled an increase in the variation of their abundances (Table 2.1). The opposite was true for generalist species, where a decrease in species number paralleled an increase in their evenness. However, both specialist and generalist evenness were found to be unaffected by isolation. The opposite trends for species richness *vs.* evenness are likely the cause of the absence of significant trends in Simpson diversity (Table 2.1).

Seven emergent groups were obtained after inspection of the different cutting levels (Table 2.2, Appendices 2.3 & 2.6). Group names were based on the groups' trait composition: Megaphanerophytes, Forest/shrub species, Orchids, Small grassland herbs, Large herbs & grasses, Sedges & shallow soil specialists and Annuals. Species richness response to restoration age and isolation differed between these seven emergent groups, with significant effects of both restoration age and isolation for 5 of the 7 emergent groups; with a decrease in species richness of Megaphanerophytes, Forest/shrub species and Large herbs & grasses and an increase in species richness of Small grassland herbs and Sedges & shallow soil specialists with progressing assembly and decreasing isolation (Table 2.1, Fig. 2.3). The significant interaction terms, however, indicate that differences in species richness of Megaphanerophytes, Forest/shrub species and Small grassland herbs caused by isolation are becoming smaller with increasing grassland age (Table 2.1). Annuals were found to increase with progressing assembly but were unaffected by isolation. Note that the significant isolation effects in Table 2.1 occurred while controlling for both soil depth and plot inclination, indicating their independence of these abiotic variables.

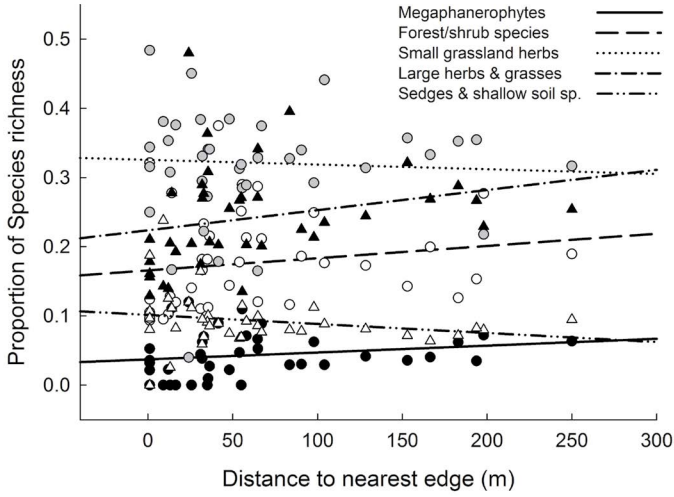


Table 2.2. Overview of the derived emergent groups. Emergent group name, typical plant traits and number of species are given.

Emergent group	Group name	Characteristics	Number of species
1	Megaphanerophytes	Long lived, early flowering, wind pollinated, large seeds, transient seed bank, allogamous, anemo- & dysochores. Species of nutrient rich soils.	14
2	Forest/shrub species	Long lived, shade-tolerant, insect pollinated, transient seed bank, mixed mating system, few & heavy seeds, dysochores, large leaves. Species of nutrient rich soils.	40
3	Orchids	Many, small seeds, mycorrhizal dependent.	13
4	Small grassland herbs	Allogamous, shade intolerant, small herbs, autochores & zoochores, nitrogen fixators, semi-rosette specialists.	68
5	Large herbs & grasses	Semi-rosette species, late flowering, large seeds, large leaves, hemero- & zoochores, competitives. Species of nutrient rich soils.	53
6	Sedges & shallow soil specialists	Mixed mating system, long seed bank longevity, small & light seeds, auto- & anemochores, mycorrhizal-independent.	28
7	Annuals	Early flowering, autogamous, short-lived, small seeds & plants, zoochores, ruderals.	31

Figure 2.3. Correlation between proportional species richness and grassland patch isolation for five Emergent groups: Megaphanerophytes (full circle, continuous line), Forest/shrub species (open circle, long dashed line), Small grassland herbs (grey circle, dotted line), Large herbs & grasses (full triangle, dash-dot line) and Sedges & shallow soil specialists (open triangle, dash-double dot line). Data points are the mean richness values for each separate grassland patch.

Analysing the β-coefficients of the logistic regression equations, we found that large plants with long seeds are more likely to occur in ancient grasslands (Table 2.3), and that large plants with large and heavy seeds and a low seed attachment potential were less likely to colonize more isolated restoration patches (Table 2.3). The filtering caused by isolation was again found to decrease with increasing time since restoration (Table 2.3). χ^2-tests showed that there was no bias in the representation of generalist or specialist species in the logistic regression analyses ($\chi^2 = 0.10$, $P = 0.75$). However, significant effects were found for the different Emergent groups, with less Orchids and Sedges & shallow soil specialists and more small grassland herbs included than excluded in the analyses ($\chi^2 = 13.76$, $P = 0.032$).

Table 2.3. Spearman rank correlations between plant traits and logistic regression β-coefficients for restoration age and isolation. Spearman R given for β-coefficient for restoration age, isolation and the interaction term (n = 108). Seed number was logarithmically transformed. Significance: *0.05 ≥ *P*-value > 0.01 **0.01 ≥ *P*-value > 0.001 ***0.001 ≥ *P*-value.

	Spearman R for β age	Spearman R for β isolation	Spearman R for β age*isolation
plant height	0.24*	0.21*	-0.20*
seed length	0.20*	0.27**	-0.29**
seed shape	0.075	0.0019	-0.020
seed longevity	-0.042	-0.12	0.11
seed mass	0.11	0.24*	-0.26**
seed number	0.070	0.017	0.0021
attachment potential sheep	-0.0071	-0.18*	0.17
attachment potential cattle	-0.15	-0.21*	0.21*

2.5 DISCUSSION

2.5.1 GENERAL ASSEMBLY PATTERNS

Whereas total species richness of the restoration patches did not change through time, we found directional community assembly when focusing on all other measures of community composition. More importantly, we could identify independent effects of spatial isolation on the assembly process, with increasing isolation delaying the community assembly process. This is largely in accordance with previous studies (Cook *et al*. 2005; Bischoff *et al*. 2009; Matthews & Endress 2010). It cannot be excluded that these assembly patterns through time are partly driven by changes in abiotic soil conditions (Piqueray *et al*. 2011b). We also observed significant autocorrelation for grassland patch isolation, indicating that certain unmeasured, spatially varying, abiotic variables might have affected the observed isolation patterns.

The 15 year old grassland plots have high leverage on our results, since a restoration time gap exists between 9 and 15 years. For this reason, the analyses were re-run after exclusion of the oldest plots. Although significance levels decreased, almost all significant patterns and conclusions remained similar, with the exception of the significant decline of generalist species through assembly.

2.5.2 SPECIES LEVEL ASSEMBLY

Overall species richness and diversity were found to be unaffected by time since restoration and isolation. Species composition was found to change with progressing assembly, as seen in the NMDS ordination scores. More specifically, the change in species composition involved the replacement of generalist species by specialist species. This change was counteracted by increasing isolation, indicating a slowdown of species assembly caused by spatial isolation.

The studied calcareous grasslands are known to lack an extensive persistent seed bank of specialist grassland species (Bossuyt *et al.* 2006), which was further confirmed in this study by the absence of a significant effect of the seed longevity index on a species' response to grassland patch isolation. This implies that species colonization is almost fully dependent upon seed dispersal, explaining the importance of spatial isolation. This results in the longer persistence of widely available generalist species, possibly extending immigration credits (defined by Jackson & Sax (2010) as the number of species committed to eventual immigration, following a forcing event such as restoration through tree removal), since specialist species were found to have low colonization potential (Pywell *et al*. 2003). Although other studies indicate that isolation effects on assembly rates can become more pronounced with progressing assembly, we observed a decrease in the isolation effects with increasing time since restoration (Cook *et al.* 2005; Fukami *et al.* 2005; Trowbridge 2007). This could indicate that in this system strong priority effects will likely not occur.

2.5.3 TRAIT LEVEL ASSEMBLY

At the emergent group level, progressing community assembly involved the gradual replacement of woody species and large competitive herbs & grasses with small stress-tolerant herbs, shallow soil specialists and annuals. The absence of a significant pattern for Orchids was expected, since these species are expected to arrive late in the community assembly process (Gijbels *et al.* 2012). The emergent group assembly was also counteracted by isolation, with increasing isolation leading to an increase in woody species and large competitive herbs & grasses and a decrease in small stress-tolerant herbs and shallow soil specialists. The absence of an isolation effect for annual species can be explained by the fact that these species are known produce a large number of highly dispersive, dormant seeds (Grime 1977).

The general direction of the emergent group assembly, however, remained unaffected by isolation, since all emergent groups were affected in the same way by restoration age as they were by isolation (with exception for Annuals, which were unaffected by isolation). This implies that although dispersal limitation clearly affects assembly at the trait level, it does not override the functionally predictable assembly mechanisms driven by the available niches (Petermann *et al.* 2010). This indicates that

it is possible to predict the community composition of restored calcareous grasslands at a certain point in time in terms of trait composition, but only when the position of the patch with respect to potential source patches is known.

It has been suggested that isolation can act as a dispersal trait filter that acts independently from restoration age (cf. Clark *et al.* 2007; Lindborg *et al.* 2011). We indeed found a higher incidence of small species with light seeds and a high seed attachment potential to both cattle hairs and sheep wool in highly isolated grasslands. These seed traits promote dispersal, suggesting that dispersal and possibly colonization are more critical in isolated grasslands. These results are in accordance with previous studies, where low seed mass was found to have a significant effect on dispersal distance, irrespective of plant height (Thomson *et al.* 2011). However, unlike previous research, we did not find a positive effect of plant height on dispersal distance, probably because our analyses are restricted to grassland species, whereas plant height effects are mainly expected for tree and shrub species (Thomson *et al*. 2011). The significant effect of attachment potential was expected, since these grasslands are managed by grazing through migrating sheep flocks, which are known to function as mobile seed vectors (Poschlod *et al*. 1998; Adriaens *et al.* 2007). When interpreting these results we have to take into account that only 44% of all present species were included, with a underrepresentation of certain Emergent groups.

2.5.4 CONCLUSION

We observed a more predictable directional community assembly at the trait level than at the species level. This indicates the importance of combining both approaches to clearly infer the restoration status of semi-natural grasslands (Pywell *et al.* 2003; Pottier *et al.* 2009; Woodcock *et al.* 2011). More importantly, we showed that spatial isolation slows down assembly at both the species and the trait level. At the trait level, this slowdown does not seem to change the predictable trajectory of assembly, with the exception of species dispersal traits, with isolated restoration patches biased towards species with a higher dispersal capacity. We did, however, observe that this slowdown of assembly caused by isolation will decrease with time for certain emergent groups. The incorporation of spatial configuration and trait based analyses into restoration schemes are therefore of high importance, since they will help to predict the outcome of ecological restoration efforts.

CHAPTER 3.

TRAIT BUT NOT SPECIES CONVERGENCE

Trait but not species convergence during plant community assembly in restored semi-natural grasslands

ADAPTED FROM:

Helsen K, Hermy M, Honnay O (2012) Trait but not species convergence during plant community assembly in restored semi-natural grasslands. *Oikos* 121: 2121-2130.

3.1 SUMMARY

Community assembly or succession was traditionally thought of as being deterministic and directional, leading to a clearly defined climax state. The alternative view, however, keeps gaining attention. This view states that community assembly is influenced by historical processes, where differences in the sequence and timing of species arrival result in distinct communities. Here we tested the hypothesis that both views are valid, but at a different level, with increasing dissimilarity in species composition among sites with increasing age (divergence), caused by historical processes (priority effects), and with increasing similarity in mean trait composition (convergence) among sites, indicating a directional development at the niche level. We surveyed a chronosequence of restored semi-natural grassland patches on former pine plantations and *Buxus* encroachment over 7 restoration age classes, covering 22 grasslands. Pairwise multivariate distances were calculated between the different grassland patches based on species abundance on the one hand, and on mean community trait values for 28 plant life history traits on the other. Trait composition showed a clear decrease in multivariate distance with increasing restoration age, indicating trait convergence through time. At the species level, we found no evidence of convergence through time, with even a trend towards divergence. Furthermore, spatial variation and environmental heterogeneity were found to remain constant through time. These results confirm our hypothesis. At the trait level, limited niches occur, only filled by species having the appropriate traits, resulting in a clear deterministic model of assembly. Species identity, on the contrary, has no role in this niche filling. The first appropriate species to reach a restoration site will be most likely the ones that get established, resulting in divergence of the species composition among restored grasslands. The identity and order of these colonizing species will, in turn, likely be governed by an interplay of small-scale site-level abiotic variation, landscape effects, historical factors and chance events, turning the community assembly outcome stochastic at the species level.

3.2 INTRODUCTION

The process of community assembly has always been controversial (e.g., Samuels & Drake 1997; Chase 2003; Shipley *et al.* 2006; Collinge & Ray 2009; Pickett *et al.* 2009). Historically, assembly was viewed as a deterministic sequential replacement of species toward a single fixed stable state ('the climax model of succession', Clements 1916). According to this view, communities converged toward a common 'structure' solely determined by their environmental conditions. The alternative views of Gleason (1927) and Diamond (1975) on the other hand, acknowledged the influence of stochasticity and history on the assembly trajectory. According to this view, priority effects, caused by variation in the sequence and timing of species arrival, are responsible for a large part of the variation in species composition between communities (Grman & Suding 2010). These priority effects occur when earlier arriving species affect the establishment, growth or reproduction of later arriving species by means of competition or soil legacies. This can in turn lead to divergence in species dominance and composition between communities even under identical environmental conditions, possibly resulting in alternative stable states (Beisner *et al.* 2003; Perry *et al.* 2003). Occurrence of these priority effects have been demonstrated both in natural systems (McCune & Allen 1985; Fastie 1995; Honnay *et al.* 2001, Collinge & Ray 2009; Grman & Suding 2010) and in mesocosm experiments (Ejrnaes *et al.* 2006; Körner *et al.* 2008).

Following many years of debate between advocates of the deterministic *versus* the contingent hypotheses of assembly (Samuels & Drake 1997; Pickett *et al.* 2009), awareness is growing that both alternatives can, and should, be considered as realistic trajectories (Young *et al.* 2001; Chase 2003; Fukami *et al.* 2005). For instance, it has been suggested that contingent community assembly is more likely to occur when the regional species pool is large, primary production is intermediate and disturbance and species dispersal rates are low (Foster 2001; Chase 2003). It has also been stated that both trajectories can occur in the same communities, but on different levels of community organization. Assembly can then be considered contingent on landscape and historical factors at the species level, but deterministic at the functional group level (Fox 1987; Kahmen & Poschlod 2004; Fukami *et al.* 2005). This hypothesis is based on the assumption that similar niches occur in communities under similar environmental conditions, leading to similar functional groups filling up the niche space within these communities (Fukami *et al.* 2005; Cleland *et al.* 2011). Assuming the occurrence of

multiple species fitting any given niche in the regional species pool, species composition within the functional groups is prone to stochasticity and priority effects, since newly arriving species will only be able to colonize communities in which their niche is not already occupied (Grime 2006).

Many authors have reported similar trait composition in different communities under equal environmental conditions, and classified it under the concept of *community* texture convergence (Matsui *et al.* 2002; Smith & Wilson 2002; Watkins & Wilson 2003; de Bello *et al.* 2009; Matthews & Spyreas 2010). Although these studies provide valuable insights into the outcome of the assembly process, they cannot inform us with respect to the process of community formation itself, since they were performed on climax state communities. To our knowledge, only one study so far has studied the process of community formation itself, by monitoring changes in species and trait composition during a nine year grassland experiment (Fukami *et al.* 2005). This experiment was initiated with clearly distinct and artificial founder communities. Nevertheless, clear convergence in trait composition occurred, whereas species composition remained differentiated (Fukami *et al.* 2005). It remains an open question, however, whether results from a controlled experimental setting can be generalized toward plant community assembly in natural systems.

The main objective of this chapter was to test the hypothesis that both succession theory and community assembly theory are valid, but at a different community level, with contingent assembly valid at the species composition level, but deterministic succession at the trait composition level. We used species abundance data of 143 plots across 22 semi-natural grasslands assembled over a seven year time span, and trait composition using 28 relevant functional plant traits. More specifically, we compared temporal changes in community dissimilarity between different grasslands in both species and trait composition using a chronosequence approach. This allowed us to test the hypothesis that community dissimilarity among grassland patches decreases at the trait level (trait convergence) but increases at the species level (species divergence) during community assembly of semi-natural grasslands.

3.3 MATERIALS AND METHODS

3.3.1 STUDY AREA

The restored grasslands were surveyed using two 2 x 2 m plots for every restored hectare (see chapter 1.4 for a detailed description of the study area). In total, 143 plots were established in 43 restoration patches (of different age, ranging from 3 to 9 years) on 22 grasslands. Note that these plots are the same as those used in chapter 2, excluding the four plots restored in 1995 (Table 1.2, Fig. 1.7). Note that the four plots restored in 1995 were excluded, since they were all part of the same grassland fragment (Table 1.2), thus resulting in no β-distances for this age group (see chapter 3.3.4). Species occurrence and cover (%) of all vascular plants (tracheophytes) were recorded in the plots during spring and early summer of 2010.

Several abiotic variables were surveyed in each plot. We measured the cover (%) of bare rock and open soil, the soil depth and plot inclination. We also calculated spatial variables for every grassland patch using QGIS 1.5.0 (Quantum GIS Development Team 2010), namely the grassland patch elevation and four metrics of spatial isolation; closest edge distance, closest centroid distance, buffer isolation and Hanski isolation, as defined in chapter 2 (2.3.2.1).

3.3.2 SPECIES TRAITS

28 plant traits were selected for trait analysis (Appendix 2.2). Traits were chosen upon their assumed relevance for community assembly, including the processes of dispersal, establishment and persistence (cf. Weiher *et al.* 1999; Violle *et al.* 2007). Trait values were extracted from several databases and sources (Fitter & Peat 1994; Thompson *et al.* 1997; Lambinon *et al.* 1998; Klotz *et al.* 2002; Poschlod *et al.* 2003; Cappers *et al.* 2006; Kleyer *et al.* 2008). The Seed longevity index was calculated according to Bekker *et al.* (1998), Seed attachment potential was calculated according to Römermann *et al.* (2005). In total, 92% of all trait values were available for all species.

For trait analysis, each vegetation sample (plot) was described in terms of the average value of each trait (community-weighted trait means CWM, $\bar{t}$) and its standard deviation (community-weighted trait standard deviations σ), according to the following formulas (Díaz *et al.* 2007; Sonnier *et al.* 2010). For every species *i* in plot *j* of grassland patch *k*, we calculated for every trait *t*: $\bar{t}_{jk} = \sum_{i=1}^{S} p_{ijk} t_i$ and $\sigma_{jk} = \sqrt{\sum_{i=1}^{S} p_{ijk} (t_{ijk} - \bar{t}_{jk})^2}$. p_{ijk} is the relative abundance of species *i* in plot *j* of grassland patch *k* and S is the total number of species in the plot. therefore quantifies the average trait value expressed by the vegetation, while quantifies the variability of this trait value around the average value within the vegetation (Sonnier *et al.* 2010). Prior to CWM calculation, nominal traits were recoded as dummy variables. All trait values were furthermore standardized to obtain a mean of zero and a standard deviation of one for every trait over all species of the species pool, before calculation of community-weighted trait means and standard deviations. This ensures that the weight was equal for all traits during subsequent analyses.

3.3.3 COMPOSITIONAL CHANGES

From the resulting trait analysis, a *plot x traits* matrix was assembled, with for every trait both community-weighted trait means and community weighted standard deviations as a matrix column. Multivariate distance matrices were constructed for both the *plot x species* matrix and the *plot x traits* matrix, to quantify the overall similarity between the different plots in terms of respectively species and trait composition. The *plot x species* matrix consisted of the relative abundances of all occurring species.

Several multivariate distance matrices were constructed for both matrices, consisting of distances between each unique pair of plots. These distances were calculated based on the Euclidean distance (D_E), the mean censored Euclidean distance (D_{MCE}) (Krebs 1989) and the Bray Curtis dissimilarity measure (D_{BC}) (Bray & Curtis 1957). Euclidean distance was used because it is conceptually the most straightforward distance measure, being the multidimensional version of the Pythagorean theorem (McCune & Mefford 1999). The mean censored Euclidean distance consists of a standardization of the

Euclidean distance; $D_{MCE} = \sqrt{\frac{(D_E)^2}{S}}$, where D_E is the Euclidean distance and S is the number of species in the vegetation plots. This metric was calculated to correct for the possible increase in Euclidean distance with increasing species number (Krebs 1989; Fukami *et al.* 2005). Both Euclidean distance and D_{MCE}, however, tend to emphasize outliers, causing them to quickly lose sensitivity with increasing heterogeneity within the dataset (McCune & Mefford 1999). For this reason we also calculated the Bray Curtis dissimilarity (also called the Czekanowski's Quantitative index), since it is unaffected by this problem. The Bray Curtis dissimilarity is the one-complement of the Sørensen similarity index; $D_{BC} = 1 - \frac{2\sum_{i=1}^{p} \min(x_{1i} - x_{2i})}{\sum_{i=1}^{p} (x_{1i} + x_{2i})}$ where x_{1i} is the abundance of species *i* in plot 1, x_{2i} the abundance of species *i* in plot 2, and *p* the total number of species recorded across both units (McCune & Mefford 1999, Anderson *et al.* 2006). Distance matrix calculations were performed with PC-ORD 5.32 (McCune & Mefford 1999) and Krebswin 0.92 (Krebs 1989). The resulting pairwise dissimilarities (distances) were grouped into two categories for both species abundance and trait abundance, namely α-distances and β-distances. α-distances were defined as the multivariate species/trait dissimilarity between two plots of the same grassland patch (and same age class). β-distances were defined as the multivariate species/trait dissimilarity between two plots of different grassland patches of the same age class. Note that these distances were derived independently from all three distance metrics, resulting in three sets of α- and β-distances.

3.3.4 STATISTICAL ANALYSIS

Convergence was defined as the decrease in species or trait multivariate distance between plots with increasing restoration age. In the same way, divergence was defined as the increase in species or trait multivariate distance between plots along the successional gradient.

To test for species or trait convergence/divergence, multivariate β-distances were related to restoration age. The relation between the multivariate β-distances and restoration age was analyzed using Spearman rank correlations. Data were clustered

in a vast number of groups (22 x 22 grassland combinations) since these distances consist of pairwise grassland combinations. This high number of grassland combinations makes analysis with a linear mixed model impossible, as the high complexity caused the model to become irresolvable, with no resulting parameter estimation. Therefore, we calculated mean multivariate β-distances for every pairwise grassland fragment combination (mostly consisting of less than 5 data points) to circumvent pseudo-replication, and calculated simple Spearman rank correlations to relate the mean β-distances with restoration age.

To test whether spatial heterogeneity within the grassland patches changed during assembly, multivariate α-distances were related to restoration age. Since α-distances were clustered within the 22 grasslands, they were analyzed using a linear mixed model, taking into account the spatial clustering of data within 22 independent groups, by including grassland identity as the random factor.

Spearman rank correlations were calculated between grassland patch age and the abiotic and spatial variables, to assess whether spatial distance or environmental variation may influence our results. For these analyses mean abiotic variable values were calculated for each grassland patch, so that all Spearman rank correlations were calculated at the grassland patch level. We also tested for the occurrence of spatial autocorrelation for grassland patch age and isolation by calculating Moran's I. All statistical analyses were performed with SAS 9.2 (SAS Institute Inc. 2004).

Table 3.1. Correlations of multivariate α- and β-distances to restoration age for both species and trait composition. For β-distances Spearman rank correlations: Spearman R given (n = 109). For α-distances linear mixed models: β-coefficient given (n = 367). D_E = Euclidean distance, D_{MCE} = mean censored Euclidean distance, D_{BC} = Bray- Curtis dissimilarity. Significance: *0.05 ≥ *P*-value > 0.01 **0.01 ≥ *P* -value > 0.001 ***0.001 ≥ *P* -value.

3.4 RESULTS

In total, we observed 247 species (Appendix 2.3). Multivariate β-distances for species composition increased (species composition diverged) through assembly when looking at Euclidean distance and mean censored Euclidean distance (Table 3.1, Fig. 3.1, D_E: P = 0.029, D_{MCE}: P = 0.009). This was, however, not the case for the Bray Curtis dissimilarity (Table 3.1, Fig. 3.2, D_{BC}: P = 0.110). At the trait level, clear convergence occurred through assembly, with significant negative correlations between the multivariate β-distances and restoration age for all distance metrics (Table 3.1, Figs 3.3 & 3.4, D_E: P < 0.001, D_{MCE}: P < 0.001, D_{BC}: P < 0.001). Note that only patterns for Euclidean distance and Bray Curtis dissimilarity are visualized (Figs 3.1 – 3.4).

α-distances showed no clear change in dissimilarity through assembly. For species composition, dissimilarity remained constant through assembly for all distance metrics (Table 3.1). Also at the trait level, dissimilarity in α-distances did not change through assembly (Table 3.1). Abiotic variables were not correlated with grassland patch age, indicating that the cover of bare rock and open soil, the soil depth and plot inclination did not significantly change with patch age (Appendix 3.1). The measured spatial variables (spatial distance, patch elevation & patch isolation) were also not correlated with grassland patch age, nor did we observe significant spatial autocorrelation for grassland patch age (Appendix 3.1).

	β-distances:	α-distances:
Distance metric	Spearman R	β coëfficiënt
Species: D_E	0.21*	1.5
Species: D_{MCE}	0.25**	0.26
Species: D_{BC}	-0.11	< -0.01
Traits: D_E	-0.50***	0.22
Traits: D_{MCE}	-0.65***	<0.01
Traits: D_{BC}	-0.48***	<0.01

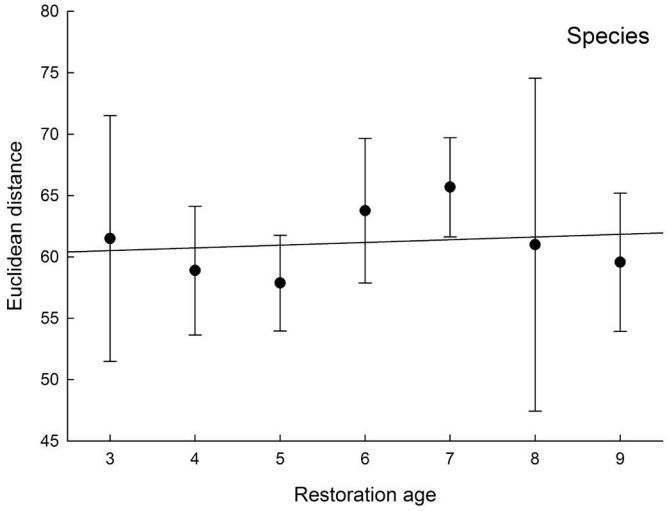


Figure 3.1. Relationship between restoration age of the grassland and the Euclidean β-distance in species composition space (r_s = 0.21, P = 0.029). Overall mean and 95% confidence interval given for every age class.

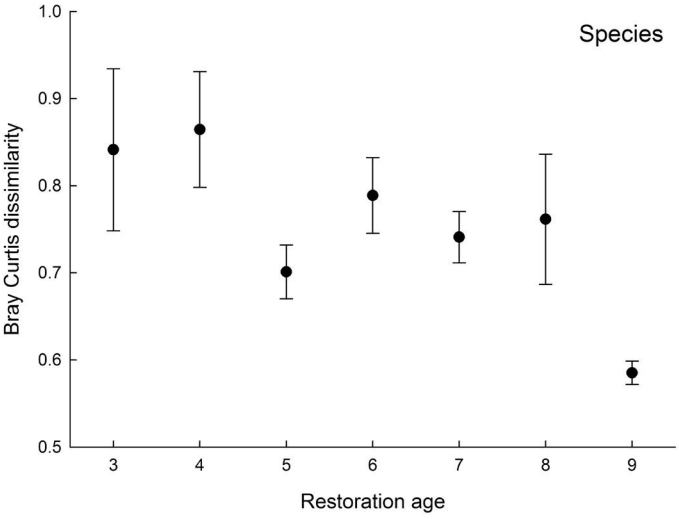


Figure 3.2. Relationship between restoration age of the grassland and the Bray Curtis dissimilarity (β-distance) in species composition space (r_s = −0.11, P = 0.24). Overall mean and 95% confidence interval given for every age class.

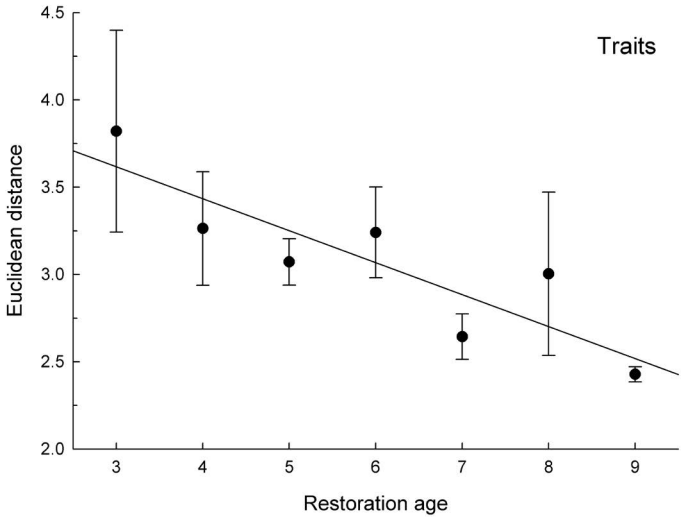


Figure 3.3. Relationship between restoration age of the grassland and the Euclidean β-distance in trait composition space (r_s = −0.49, P < 0.001). Overall mean and 95% confidence interval given for every age class.

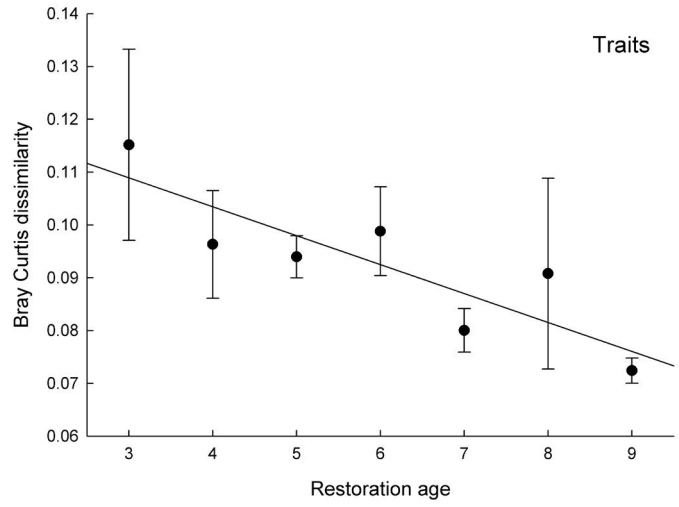


Figure 3.4. Relationship between restoration age of the grassland and the Bray Curtis dissimilarity (β-distance) in trait composition space (r_s = −0.48, P < 0.001). Overall mean and 95% confidence interval given for every age class.

3.5 DISCUSSION

We studied the community assembly of semi-natural grasslands over a period of seven years. Distance trajectories of community assembly were clearly different for species composition *versus* trait composition. Differentiation in species composition between grassland patches was found to remain constant through time, or even to increase, depending upon the used measure. Trait composition on the other hand was found to converge during community assembly between grassland patches, forming the first proof of the trait convergence hypothesis in a natural community.

These results suggest that initial differences in species composition between restoration sites are maintained throughout assembly, resulting in an absence of species convergence through time. These differences in species composition are likely caused by initial differences in the identity and order of colonizing species after restoration. With several species available for every niche in the regional species pool, species identity per se has no role in the niche filling (van der Maarel & Sykes 1993). Therefore, the first appropriate species to reach the restoration site will be more likely to get established in its niche, possibly imposing priority effects on later arriving species that fit the same niche, in turn overwriting any predicted assembly outcome (Petermann *et al.* 2010). These initial differences in species colonization are likely contingent upon (environmental) site-level conditions, landscape effects and/or historical factors (Brudvig 2011). Indeed, it cannot be excluded that small-scale variation in abiotic conditions among grassland patches could have resulted in different preferential initial colonization among patches. Spatial isolation has furthermore been observed to affect species dispersal, and can thus be expected to alter species' colonization order (chapter 2). Habitat type and accompanied species composition of the surrounding landscape (landscape matrix) can also be expected to affect initial colonization patterns (Matthews *et al*. 2009; Öckinger *et al*. 2012; Grman *et al.* 2013). Historical effects such as year effects during restoration or remnant seed bank seeds present at the patch can also be expected to change colonization patterns (Bakker *et al.* 2003; Vaughn & Young 2010). Some portion of the colonization process can alternatively be expected to be purely stochastic, with colonization of certain species partly occurring by chance (Grman *et al*. 2013). The persistence of these initial differences in species composition through assembly can alternatively (or partly) be explained by persisting dispersal limitation for certain species in isolated patches.

It is known that Euclidean distance and D_{MCE} are prone to loss of sensitivity with increasing data heterogeneity. It is therefore possible that the significant divergence for species composition is a side effect of this weakness, since no divergence was found for the Bray-Curtis dissimilarity. This has, however, little effect on our conclusions, since constant dissimilarity in species composition still indicates the absence of deterministic assembly on the species level. Furthermore, spatial isolation and the measured abiotic variables did not co-vary with restoration age, indicating that changes in β-distances among restoration age classes were mainly effectuated by differences in restoration age, rather than by confounding factors, confirming that a chronosequence approach was appropriate.

In contrast to species composition, trait composition between grassland patches clearly converged with increasing restoration time. We argue that this convergence is caused by the progressing filling of available niches within the community. We interpret the initial difference in trait composition between the grasslands at the beginning of the colonization process as the result of the random filling of only a subset of niches within these communities. This underlies the assumption that initial communities contain a significant number of unoccupied niches and that environmental filtering allows only species with traits compatible with the prevailing environmental conditions (Sonnier *et al.* 2010). If this environmental filtering is indeed driving trait assembly patterns, we can expect the occurrence of *local* trait convergence or trait clustering (Cornwell *et al.* 2006; De Bello *et al.* 2013). Evaluating our dataset for this local trait clustering would form a test of this environmental filtering hypothesis.

This community convergence on the trait level is in accordance with the experimental findings of Fukami *et al.* (2005), who concluded that the assembly on the trait level is governed by deterministic trait-based assembly rules, caused by the filling of a limited number of niches available in the grassland. Although clear deterministic trait-based community assembly in natural systems has not been demonstrated so far, several studies have found evidence for the following assembly rule: 'Each species entering a community is likely to be drawn from a different functional group until each group is represented, and then the rule repeats' (Fox 1987; Wilson & Roxburgh 1994; Wilson & Whittaker 1995; Belya & Lancaster 1999). This suggests the existence of deterministic trait-based community assembly, further confirming our results. The theory of limited similarity of co-occurring species also indicates that niche occupancy is likely important for community assembly (Stubbs & Wilson 2004; Schamp *et al.* 2008; De Bello *et al.* 2009; Pillar *et al.* 2009).

We opted to use community-weighted trait means instead of clustered functional trait groups. This is because the use of functional trait groups has recently been criticized as being necessarily subjective, since the delineation of separate trait groups from the dendrogram remains arbitrary, and often leads to a substantial loss of trait variation in the dataset (Shipley 2010). By directly dealing with the traits themselves these problems are circumvented.

In contrast to the multivariate distances *among* grassland patches, we found that multivariate distances among plots *within* the grassland patches remained constant through time, for both species and trait composition. These distances reflect the spatial variation in species and trait composition at the grassland patch scale. Therefore, these results suggest that spatial heterogeneity remains constant throughout assembly and no divergence occurs between local micro patches within a grassland age patch at the studied scale.

Restoration ecology has not always paid attention to the occurrence of contingencies (Young *et al.* 2005; Brudvig 2011). Our results, however, suggest that contingencies caused by landscape or historical factors may play an important role in the restoration of grasslands. We argue that conservation and restoration research should think more in terms of traits in predicting or anticipating the results of certain restoration practices (Klimkowska *et al.* 2010; Sandel *et al.* 2011). We also believe that ecological restoration projects can only lead to desired results if the target niche is not filled by undesired species before the target species presents itself on the site. This suggests that restoration projects should not focus on the conservation of single species. Taking into account trait assembly rules could help the design of better restoration plans, or explain unexpected vegetation patterns after community assembly (Suding *et al.* 2004).

In this study we observed clear convergence of trait composition, whereas species composition did not converge, with even a trend towards divergence. These results reflect the persistence of initial differences in colonizing species composition, possibly through priority effects or persistent dispersal limitation. To our knowledge, this work represents the first study finding evidence for the species divergence – trait convergence hypothesis in a natural landscape. Field observation studies, as this one, form valuable additions to experimental approaches. More assembly studies in natural communities, covering a larger range of restoration ages, are necessary however.

CHAPTER 4.

GENETIC DIVERSITY IN FOUNDER POPULATIONS

Rapid buildup of genetic diversity in founder populations of the gynodioecious plant species *Origanum vulgare* after semi-natural grassland restoration

ADAPTED FROM:

Helsen K, Jacquemyn H, Hermy M, Vandepitte K, Honnay O (2013) Rapid buildup of genetic diversity in founder populations of the gynodioecious plant species *Origanum vulgare* after semi-natural grassland restoration. *Plos One* 8:e67255.

4.1 SUMMARY

In most landscapes the success of habitat restoration is largely dependent on spontaneous colonization of plant species. This colonization process, and the outcome of restoration practices, can only be considered successful if the genetic makeup of founding populations is not eroded through founder effects and subsequent genetic drift. Here we used ten microsatellite markers to investigate the genetic effects of recent colonization of the long-lived gynodioecious species *Origanum vulgare* in restored semi-natural grassland patches. We compared the genetic diversity and differentiation of fourteen recent populations with that of thirteen old, putative source populations, and we evaluated the effects of spatial configuration of the populations on colonization patterns. We did not observe decreased genetic diversity in recent populations, or inflated genetic differentiation among them. Nevertheless, a significantly higher inbreeding coefficient was observed in recent populations, although this was not associated with negative effects on two measured proxies related to reproductive success. Overall population genetic differentiation was low (F_{ST} = 0.040). Individuals of restored populations were assigned to on average 6.1 different source populations (likely following the 'migrant pool' model). Gene flow was, however, affected by the spatial configuration of the grasslands, with gene flow into the recent populations mainly originating from nearby source populations. This study demonstrates how spontaneous colonization after habitat restoration can lead to viable populations in a relatively short time, overcoming pronounced founder effects, when several source populations are nearby. Restored populations can therefore rapidly act as stepping stones and sources of genetic diversity, likely increasing overall metapopulation viability of the study species.

4.2 INTRODUCTION

In many landscapes, large scale habitat restoration has proven to be the only way to establish self-sustaining ecosystems that are resilient to future perturbation (Rice & Emery 2003; Rey Benayas *et al.* 2009). The success of these restoration schemes always depends to some extent on spontaneous colonization of the newly created habitats by plant species. The long term viability of these newly established populations and even communities, however, can be expected to be heavily dependent on their genetic makeup (Lande 1988; Jamieson & Allendorf 2012; Reynolds *et al.* 2012). Therefore, it is of high importance to get insight into the processes that affect genetic diversity of plant populations that have colonized restored habitats (Montalvo *et al.* 1997). So far, there are only few reports on the genetic effects of spontaneous plant colonization directly following ecological restoration practices (e.g. Travis *et al.* 2002; Van Looy *et al.* 2009; Vandepitte *et al.* 2012).

Metapopulation genetic theory can help to understand the potentially complex genetic consequences of early colonization following habitat restoration (Slatkin 1977). Since colonization often involves the establishment of a limited number of founding individuals, only a subsample of the genetic variability of the source populations will be present in colonizing populations. These founder effects, or genetic bottlenecks, reduce local population genetic diversity and can result in large genetic differentiation between colonizing populations, especially when population growth rates remain small after colonization (Nei *et al.* 1975; McCauley 1991). In the same way, these bottlenecks can cause an increase in non-random associations between pairs of loci (linkage disequilibrium), which in turn may accelerate stochastic loss of genetic diversity when random genetic drift due to small initial population sizes persists in these founder populations (Zartman *et al.* 2006; Honnay *et al.* 2009).

To what extent founder events reduce population genetic diversity and increase the magnitude of genetic differentiation among populations is dependent on (i) the number of colonizing individuals that arrive in the new habitat, relative to the number of migrating individuals between extant populations, and (ii) the degree of common source population origin of colonizing propagules (Slatkin 1977; Wade & McCauley 1988; Whitlock & McCauley 1990; Ingvarsson 1997; Pannell & Charlesworth 2000). In general, genetic founder effects are predicted to be strong when population establishment is mediated

by few colonists from a limited number of source populations (the 'propagule pool' model). Founder effects are predicted to be weak or even absent, on the other hand, when colonization occurs from multiple source populations (the 'migrant pool' model) (Slatkin 1977; Pannell & Charlesworth 1999). The probability (φ) that two colonizing individuals originate from the same source population (with $\varphi = 1$ for the propagule pool model and $\varphi = 0$ for the migrant pool model) is therefore a good indication of the extent of founder effects (Whitlock & McCauley 1990). Increased genetic differentiation between founder populations is expected if the inequality $k < \frac{2Nm}{1-\varphi} + 0.5$ holds, with *k* the number of colonists and *Nm* the effective number of migrants (Whitlock & McCauley 1990; Pannell & Charlesworth 2000).

As the extent of genetic founder effects is strongly mediated by the amount and the direction of gene flow between populations, it can be expected to depend strongly on the spatial configuration and the connectivity of the restored populations and the source populations (Sork & Smouse 2006). Limited founder effects can be expected, for instance, when many source populations are present that are well connected to the recent populations, either by small geographical distance, a permeable landscape matrix, a high intrinsic potential for gene flow of the colonizing species, or a combination of any of these factors (Giles & Goudet 1997; Austerlitz *et al.* 2000). Taking into account the position of possible source populations, relative to restored habitat patches, will therefore help to better understand the temporal and spatial patterns of genetic diversity in restored plant populations, possibly resulting in clearer guidelines for future ecological restoration schemes (Huxel & Hastings 1999; Holderegger *et al.* 2010; Segelbacher *et al.* 2010).

In this chapter we investigated the genetic consequences of recent colonization of the long-lived plant species *Origanum vulgare* (*Lamiaceae*) in newly restored semi-natural grassland patches. This species is a diploid (2*n* = 30), aromatic perennial herb, mainly occurring in grasslands on relatively dry, nutrient-poor to moderately nutrient-rich calcareous soils (Lambinon *et al.* 1998). Individual plants can live up to 5-50 years and flowering can occur a year after germination, during summer and early fall (Klotz *et al.* 2002; Kleyer *et al.* 2008). The species is self-compatible, but mainly outcrossing (facultative allogamous) due to protandry, and is pollinated by insects, mainly bees and bumblebees (Klotz *et al.* 2002). *Origanum vulgare* exhibits gynodioecy, in which both hermaphroditic and functionally female individuals co-occur (Barrett 2002, Bailey & Delph 2007). Female plants of *O. vulgare* bare exclusively male-sterile flowers, which

contain one receptive stigma and four aborted (sterile) anthers and are considerably smaller than those of hermaphroditic plants (Ietswaart *et al.* 1984). The sex ratio of natural populations of *O. vulgare* in western Europe has been found to vary between 1-62% of male sterility (Kheyr-Pour 1980). Propagation is accomplished by a large number of small seeds (mean weight 0.1 mg), which germinate in vegetation gaps during spring. Seed dispersal occurs through autochory, anemochory and epizoochory (Klotz *et al.* 2002). The seeds can form a persistent seed bank, with a seed bank longevity index (LI) of 0.41 *sensu* (Thompson *et al.* 1998), indicating that *O. vulgare* was classified as forming a persistent seed bank in 41% of all performed seed bank studies including this species (Klotz *et al.* 2002). In only 23% of the studies observing persistent seeds for *O. vulgare*, seeds were found to survive longer than 5 years in the soil (Klotz *et al.* 2002). Vegetative reproduction also occurs through a rhizome-like pleiocorm.

To investigate the genetic consequences of the recent colonization of *Origanum vulgare*, we compared genetic diversity, linkage disequilibrium and genetic differentiation between fourteen recently colonized populations and thirteen old established populations, using ten highly polymorphic microsatellite markers. Since we sampled a large number of possible source populations, we were able to investigate whether the spatial configuration of source populations, relative to the colonizing populations, mediated patterns of gene flow between old and recent populations, and influenced the degree of genetic differentiation among recent populations. Because our study system consisted of a large set of recently restored grassland patches, all founder populations were of relatively recent origin (< 10 years old). This allowed us to examine the impact of source population genetic diversity on the genetic makeup of the recent populations, after only very limited effects of possible drift and inbreeding effects, which are expected to become more likely with increasing number of generations and thus with aging (Honnay 2013). Many other studies have been unable to disentangle these effects, since the study system included older founder populations (Tremetsberger *et al.* 2003; Yang *et al.* 2008; Jacquemyn *et al.* 2009).

The sex ratio in natural populations of gynodioecious plant species can be highly variable, particularly in recently established populations, due to random sampling effects during colonization (Nilsson & Ågren 2006; Bailey & Delph 2007). Skewed sex ratios can have a major impact on patterns of genetic diversity, for example by promoting cross-fertilization and therefore enhancing population genetic diversity and reducing the severity of occurring founder effects or by limiting fertilization when recent

populations are dominated by female plants, in turn enhancing the severity of occurring founder effects (Lewis & Crowe 1956; Manicacci *et al.* 1996; De Cauwer *et al.* 2012; Dufay & Billard 2012). For this reason sex ratio was included as a possible explanatory variable of population genetic diversity and structure. More specifically we asked the following questions:

1. Does the spatial configuration of old relative to recent populations of *Origanum vulgare* structure genetic colonization patterns?

2. Are recent populations of *O. vulgare* characterized by impoverished genetic diversity, increased linkage disequilibrium and inflated genetic differentiation, caused by founder events?

3. Do our results concur with either the 'propagule pool' or the 'migrant pool' model of Slatkin (1977)?

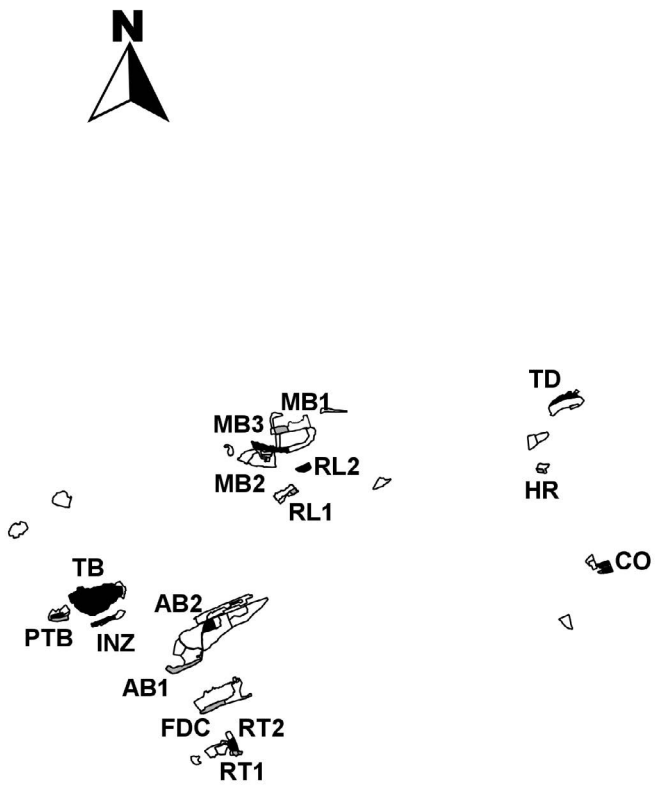
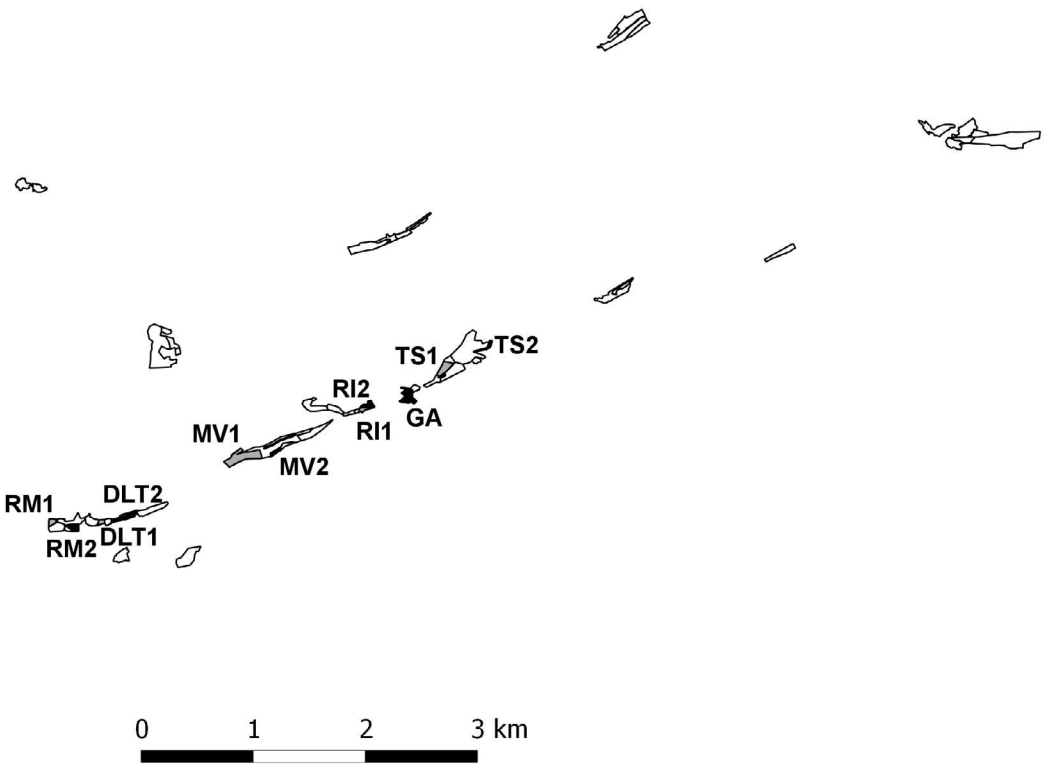


Figure 4.1. Study area in the Viroin valley. Figure visualises the sampled recent populations (grey) and old populations (black) of *O. vulgare*. Population codes correspond to those in Table 4.1.

4.3 MATERIALS AND METHODS

4.3.1 SAMPLING AND LABORATORY PROCEDURES

In our study area, *O. vulgare* occurs on both remnant and restored calcareous grasslands and in some parts of the landscape matrix, such as certain road verges and forest edges (see chapter 1.4 for a detailed description of the study area). *Origanum vulgare* responded very quickly to restoration practices and was successful at establishing large populations on several recently restored grasslands. In total, we randomly selected 27 populations: 14 populations were located in recently restored calcareous grasslands, and 13 populations in mature calcareous grasslands (Fig. 4.1). In the summer of 2011, leaf material of 20 randomly selected individuals per population was collected and dried on silica gel. For each population, population size and the percentage of female plants were determined by counting the number of female and



hermaphroditic individuals. For the largest populations (>1500 individuals) only a part of the population was counted and an approximation of the total populations size was extrapolated from this counted subset. Seed material of 25 plants per population was collected in October of 2011 and pooled per sampling location.

In the laboratory mean seed weight per population was obtained using a high precision balance (accuracy = 0.001 g). For each population, three replicates of 20 seeds were placed on moist Whatman paper in separate 10-mm Petri dishes. Seeds were incubated at 5ºC for 2 weeks, followed by 4 weeks at 20ºC with a 12h photoperiod. The number of germinated seeds was counted daily. Two proxies related to reproductive success were quantified per population: mean seed weight and the percentage of germinated seeds (germination rate) at the end of the experiment.

We extracted DNA from collected leaf samples using the Nucleospin DNA-extraction kit (Macherey Nagel, Germany). DNA quality and concentration were estimated using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For this study we used ten microsatellites developed by Novak *et al.* (2008) (OR 10, 12-14, 27, 40, 44, 64, 75 & 77). Amplifications of the DNA were carried out in two multiplexes of five microsatellites using dyeset DS-33 (Applied Biosystems, CA, USA) in 10 µL reactions containing 1 µL template DNA, 2 µL of one of the two multiplexed primer combinations (both forward and reverse primers, 1 µM), 5 µL Qiagen Multiplex PCR Master Mix and 2 µL RNAse-free water. The polymerase chain reaction (PCR) was performed using a 2720 Thermal Cycler (Applied Biosystems, CA, USA). The PCR cycling profile of Novak *et al.* (2008) was used, starting with an initial denaturation at 95°C for 15 min, followed by 35 cycles of 1 min at 95°C, 1 min at 59°C and 2 min at 72°C, with a final extension step of 9 min at 72°C. After PCR, 1 mL of reaction was added to a solution of 8.8 mL formamide and 0.2 mL of the Applied Biosystems' GeneScan 500 LIZ size standard. Fragments were sized on an ABI Prism, 3130 Genetic Analyzer (Applied Biosystems) and scored with GeneMapper Software v4.0 (Applied Biosystems).

4.3.2 DATA ANALYSIS

After checking the microsatellite data for scoring errors due to stutter bands, null alleles and large allele dropout with MICRO-CHECKER (Van Oosterhout *et al.* 2004), the mean number of alleles per population (A), expected heterozygosity (H_E) and observed heterozygosity (H_O) were calculated for each population using GenAlEx 6.5 (Peakall & Smouse 2006). The inbreeding coefficient (F_{IS}) was estimated based on Wright`s F-statistics with GenAlEx 6.5 (Peakall & Smouse 2006). We tested for the occurrence of composite linkage disequilibrium between each pair of loci in each population with exact probability tests in Genepop 4.0.10 (Raymond & Rousset 1995). This test applies Markov chain algorithms on all contingency tables corresponding to all possible pairs of loci within each population. For each population, we summed the number of allele pairs for which significant linkage disequilibrium occurred. We used this metric as an indication of severity of linkage disequilibrium (LD) for each population. We then tested for the occurrence of recent bottleneck events in each population by looking for evidence of excess heterozygosity relative to allele numbers using the Bottleneck software (Cornuet & Luikart 1996). We used a two-phase model of mutation (TPM) with a 90% stepwise component, which is considered most appropriate for microsatellite data (Cornuet & Luikart 1996).

To test for the occurrence of founder effects on the different metrics of within population genetic diversity we used first-order factorial general linear models (GLM) in Statistica 10 (Statsoft 2000) using A, H_O, H_E, F_{IS} and LD as dependent variables. The models contained population age (recent vs. old) as a factor, and population size and the percentage of female plants as covariates and all first order interaction terms. All non-significant terms were removed using stepwise model reduction to obtain the final models. Population size and the percentage of female plants (%F) were log transformed to obtain homogeneity of the variances. We tested for effects of population age and population size on the percentage of female flowers with an analogue GLM model. We also performed a Levene's test to test for differences in variance in the percentage of female flowers between recent and old populations. Mean population size was compared between restored and old populations with a t-test. To test for founder effects at the level of reproductive success, mean seed weight and germination rate were correlated to F_{IS} using Pearson correlations and compared between recent and old populations using a t-test.

Pairwise genetic differentiation among populations based on Wright`s F-statistics (F_{ST}) was calculated. Because genetic differentiation measured by F_{ST} may be underestimated for multi-allelic markers, such as microsatellites (Meirmans & Hedrick 2011), we also calculated Hedrick's G'_{ST} and Jost's D which are not affected by marker variability. G'_{ST} is the original G_{ST} as defined by Nei (1973) standardized by the maximum value it can obtain ($G_{ST(max)}$) (Hedrick 2005). Jost's D is calculated based on the effective number of alleles instead of heterozygosity, which is considered a more intuitive diversity estimate (Jost 2008). All three pairwise genetic differentiation metrics were calculated and their significance was inferred based on 9999 permutations in GenAlEx 6.5 (Peakall & Smouse 2006).

Genetic differentiation among populations was compared between recent and old populations based on a 2-tailed t-test on the pairwise $F_{ST,}$ G'_{ST} and Jost's D values. Because of the dependence of pairwise data, a bootstrapping procedure of 9999 bootstraps was applied for the calculation of the test statistics and the mean values and 95% confidence intervals for recent and old populations separately (SPSS Statistics 19.0). An analogous analysis was performed to compare pairwise geographical distances between recent and old populations. Total genetic diversity was partitioned among recent and old populations (among groups), among populations and within populations by performing a hierarchical analysis of molecular variance (AMOVA) on F_{ST} with GenAlEx 6.5 (Peakall & Smouse 2006). Significance of these genetic differentiations was tested based on 9999 permutations. Isolation by distance was tested for all populations and for recent and old populations separately, by regressing pairwise genetic (F_{ST}, G'_{ST} and Jost's D) distances on pairwise logarithmic spatial distances using Mantel tests in GenAlEx 6.5 (Peakall & Smouse 2006). A total of 9999 random permutations were performed. Geographical distances between populations were calculated as the Euclidean distance between population centroids using QGIS 1.7.4 (Quantum GIS Development Team 2010).

To characterize the overall genetic structure, we applied a Bayesian clustering approach implemented in STRUCTURE 2.3.3 (Pritchard *et al.* 2000). We used the admixture model with correlated alleles applying burn-ins of 10^4 and runs of 10^5 repetitions for each value of *K* (number of population clusters), varying from 1 to 16. We performed 20 iterations for each tested value of *K*. The entire model was also rerun using the LOCPRIOR option. This allows the model to use population identity of the individuals as prior information to assist the clustering. The true number of *K* for both models was identified based on the approach of Evanno *et al.* (2005).

Finally, to infer colonization patterns, individual plants of recent populations were assigned to old populations based on the Monte Carlo resampling procedure (Rannala & Mountain 1997) implemented in GeneClass2 (Piry *et al.* 2004). This procedure makes use of the allele frequency distributions, using a Bayesian approach to assign individuals, with assignment probabilities based on a threshold of $P = 0.05$. Based on the assignment analysis, we calculated φ_p for each recent population, i.e. the probability that two gene flow events into a recent population originate from the same source population, by combinations of probabilities (Honnay *et al.* 2009). The φ_p for each recent population was calculated as $\sum_{s=1}^{n}\left(\frac{k_s}{k_t}\times\frac{k_s-1}{k_t-1}\right)$, with s the number of source populations of which plants originated in the recent population p (with $p = 1$ to m), k_s the number of plants in the recent population p genetically originating from source population s (with $s = 1$ to n) and k_t the total number of plants in the recent population (in this case the number of sampled individuals in each recent population, $k_t = 20$). The overall value of φ_p for the study system was calculated as the mean φ_p over all recent populations. To infer the effects of spatial configuration on gene flow, we correlated the geographical distance between each recent population and the different source populations, with the number of plant individuals assigned to each of these source populations. Since these data points are not independent, we performed a linear regression with bootstrapping (10000 bootstraps) (SPSS Statistics 19.0).

4.4 RESULTS

4.4.1 GENETIC DIVERSITY

MICRO-CHECKER results indicated no problems with scoring errors due to stutters or allelic dropout in any of the 10 loci. We did, however, detect the occurrence of a homozygote excess in 22% of the populations for 2 loci (OR 12 and OR 75), possibly indicating the occurrence of null alleles. For this reason, all analyses were rerun excluding these loci. As the obtained results were similar to those obtained using all 10 loci, we decided to include all 10 loci in the analyses.

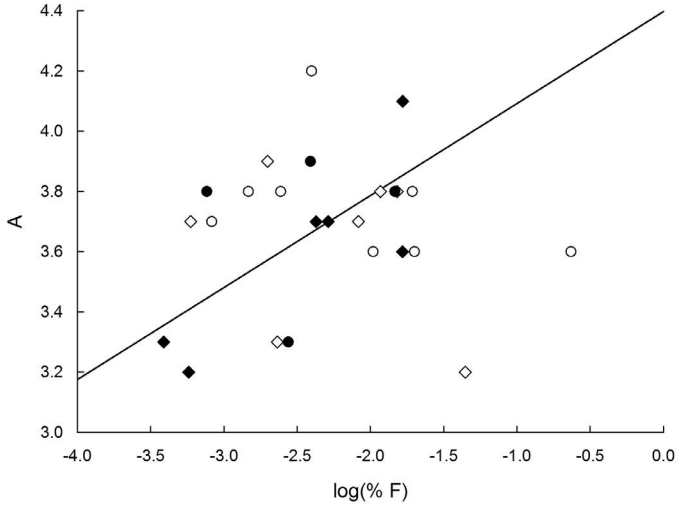
Recent populations had a median of 708 plants (range: 150-5000), with median of 9.7% female plants (range: 3.3-25.9%). The number of alleles per population (A) varied between 3.1 and 4.1 alleles per population (average: 3.6), whereas observed heterozygosity varied between 0.37 and 0.48 (average: 0.42) for recent populations (Table 4.1). Old populations had a median of 9.1% female plants (range: 4.6-53.2%) with a median population size of 459 plants (range: 99-2250), a mean A of 3.7 (range: 3.3-4.2) and a mean H_O of 0.44 (range: 0.41-0.54) (Table 4.1). No significant difference between recent and old populations was found for population size (t = 1.0, P = 0.31), the percentage of female flowers (t = -2.2, P = 0.56) or the variance in the percentage of female flowers (F = 1.2, P = 0.76). The percentage of female flowers was not related to population size. The mean number of alleles increased with increasing percentage of female plants. However, this pattern was influenced by population size, with a decrease in the positive correlation with decreasing population size (significant interaction term, Table 4.2). This was visualized by dividing population size in small (<500 plants) and large populations (>1000 plants), and performing a Pearson correlation test independently for small and large populations (Fig. 4.2). Both H_O and F_{IS} were affected by population age, with a significantly lower observed heterozygosity and higher inbreeding in recent populations (Table 4.2, Fig. 4.3). Expected heterozygosity (H_E) was not affected by any of the measured population characteristics. Linkage disequilibrium (LD) on the other hand, decreased with increasing population size, but was unaffected by population age (Table 4.2). When we tested for a correlation between LD and population size for recent and old populations independently, we found a significant correlation for old populations (r = -0.61, P = 0.027), but not for recent populations (r = -0.43, P = 0.12). We found no evidence of recent genetic bottlenecks in any of the 27 populations. Reproductive success was not affected by F_{IS} (germination rate: r = 0.025, P = 0.90; seed weight: r = -0.084, P = 0.68) or population age (germination rate: t = -1.068, P = 0.30; seed weight: t = 0.26, P = 0.80).

Table 4.1. Population characteristics of all sampled *Origanum vulgare* populations for recent and old populations separately. %F: percentage of female plants in the population; A: the mean number of alleles per population; H_E: the expected heterozygosity; H_O: the observed heterozygosity; F_{IS}: the approximated inbreeding coefficient, LD: number of allele pairs for which linkage disequilibrium occurred and SD: standard deviation.

Recent population	code	pop. size	%F	A	H_E	H_O	F_{IS}	LD
Les Abannets	AB1	3000	16.9	3.6	0.45	0.39	0.13	1
Dessous le Transoi	DLT1	348	12.5	3.7	0.49	0.48	0.058	0
Fondry des Chiens	FDC	2000	3.3	3.3	0.47	0.37	0.19	1
Haute Roche	HR	923	8.0	3.1	0.40	0.37	0.014	1
Inzevaux	INZ	298	14.5	3.8	0.45	0.43	0.021	0
Montagne-aux-Buis	MB1	1088	9.3	3.7	0.48	0.43	0.12	2
Montagne-aux-Buis	MB2	250	16.3	3.8	0.51	0.43	0.11	2
Mwène à Vaucelles	MV1	5000	16.9	4.1	0.48	0.39	0.19	0
Petit Breumont	PTB	491	4.0	3.7	0.46	0.38	0.14	3
Rivelottes	RI1	150	25.9	3.2	0.42	0.40	0.036	5
Roche à Lomme	RL1	1250	10.1	3.7	0.46	0.46	-0.021	2
Roche Madoux	RM1	198	6.7	3.9	0.46	0.43	0.069	2
Roche Trouée	RT1	492	7.2	3.3	0.49	0.41	0.14	2
Tienne Saumières	TS1	2250	3.9	3.2	0.45	0.45	-0.023	2
Mean (±SD)		1267 (±1386.9)	11.1 (±6.4)	3.6 (±0.30)	0.46 (±0.029)	0.42 (±0.034)	0.084 (±0.072)	1.6 (±1.3)
Old population	code	pop. size	%F	A	H_E	H_O	F_{IS}	LD
Les Abannets	AB2	261	7.3	3.8	0.45	0.51	-0.11	1
Contienau	CO	2250	9.0	3.9	0.53	0.53	0.021	1
Dessous le Transoi	DLT2	701	13.7	3.6	0.47	0.54	-0.15	3
Gayi	GA	1500	16.0	3.8	0.47	0.49	-0.040	1
Montagne-aux-Buis	MB3	478	9.1	4.2	0.52	0.44	0.13	2
Mwène à Vaucelles	MV2	282	18.3	3.6	0.48	0.43	0.042	3
Rivelottes	RI2	443	13.8	3.6	0.50	0.46	0.092	2
Roche à Lomme	RL2	1325	4.4	3.8	0.48	0.45	0.051	0
Roche Madoux	RM2	1300	7.7	3.3	0.43	0.42	-0.014	3
Roche Trouée	RT2	110	5.9	3.8	0.51	0.49	0.027	6
Tienne Breumont	TB	99	53.2	3.6	0.42	0.46	-0.079	2
Tienne Delvaux	TD	147	4.6	3.7	0.46	0.42	0.092	7
Tienne Saumières	TS2	459	18.0	3.8	0.48	0.41	0.13	3
Mean (±SD)		720 (±667.3)	13.9 (±12.8)	3.7 (±0.21)	0.48 (±0.033)	0.47 (±0.043)	0.015 (±0.089)	2.6 (±2.0)
overall mean (±SD)		1003 (±1115.8)	12.5 (±9.9)	3.7 (±0.27)	0.47 (±0.031)	0.44 (±0.046)	0.050 (±0.087)	2.1 (±1.7)

Table 4.2. Parameter estimates of the final GLM analyses after model reduction (n = 27). A: mean number of alleles per population; H_O: observed heterozygosity; F_{IS}: the approximated inbreeding coefficient. LD: number of allele pairs for which linkage disequilibrium occurred and %F: percentage of female plants in the population. Significance: *0.05 ≥ *P*-value > 0.01 **0.01 ≥ *P*-value > 0.001 ***0.001 ≥ *P*-value.

	Model	age		pop size		%F		pop size*%F	
	R^2	F	β	F	β	F	β	F	β
A	0.15	-	-	6.4	0.35*	6.3	-0.92*	7.3	0.16*
H_O	0.27	10.7	-0.025**	-	-	-	-	-	-
F_{IS}	0.13	4.8	0.068*	-	-	-	-	-	-
LD	0.28	-	-	10.9	-0.87**	-	-	-	-



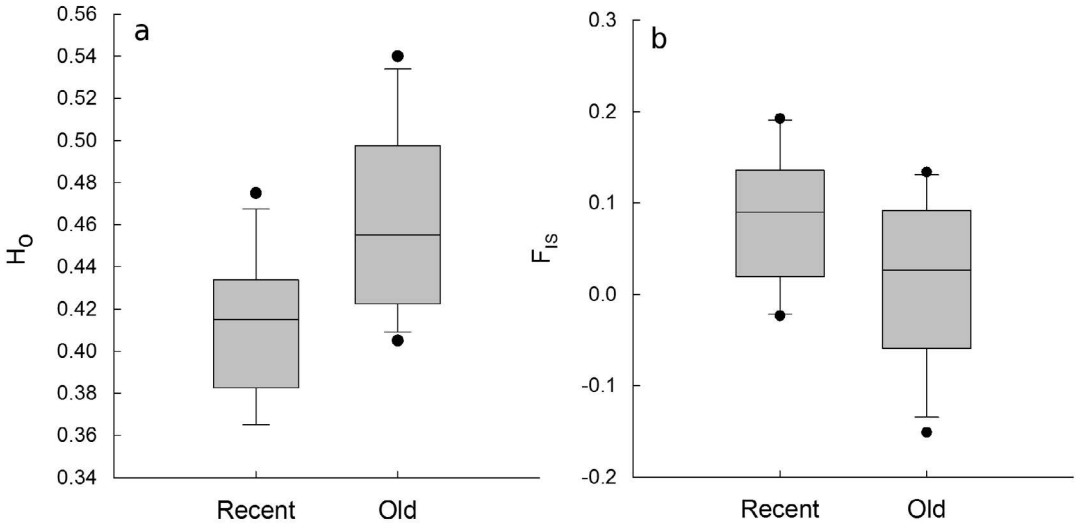


Figure 4.3. Difference in observed heterozygosity and inbreeding coefficient between recent and old populations. a. Boxplot for observed heterozygosity (H_O). b. Boxplot for inbreeding coefficient (F_{IS}).

Figure 4.2. Relation between the mean number of alleles (A) and the percentage of female plants (%F). The relation is visualized independent for small (<500 plants, open symbol, no regression line shown) (r = −0.29, P = 0.29) and large populations (>1000 plants, full symbol, continuous line) (r = 0.64, P = 0.048). Recent populations are presented as diamonds, old populations as circles. %F was log transformed.

4.4.2 GENETIC DIFFERENTIATION

The overall genetic differentiation among all populations was low (F_{ST} = 0.040, G'_{ST} = 0.058, Jost's D = 0.039), but significant according to the AMOVA based on F_{ST} (*P* < 0.001) (Appendices 4.1 & 4.2). Genetic differentiation based on F_{ST}, G'_{ST} and Jost's D was significantly higher for old populations than for recent populations (Table 4.3). The average geographic distance separating populations, however, was not significantly different between old and recent populations (Table 4.3). The AMOVA results indicated that no significant overall genetic differentiation occurred between recent and old populations (F_{RT} < 0.0001, *P* = 0.55). We observed significant isolation by distance based on F_{ST}, G'_{ST} and Jost's D when including all populations, a trend that was even stronger when only including old populations. However, no isolation by distance was observed when including only recent populations (Fig. 4.4).

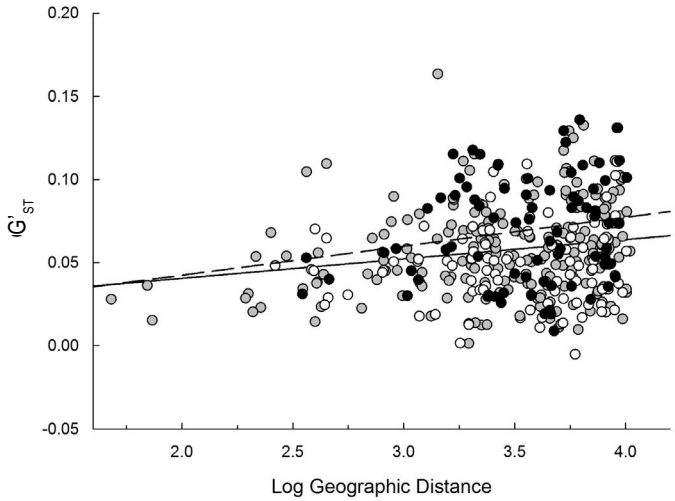


Table 4.3. Parameter estimates of performed bootstrapping analysis on pairwise differentiation for recent and old populations and differences between recent and old populations tested based on bootstrap t-tests (n = 168). All tests are based on 9999 bootstraps. CI: 95% confidence intervals. Significance: *0.05 ≥ *P*-value > 0.01 **0.01 ≥ *P*-value > 0.001 ***0.001 ≥ *P*-value.

	recent pop.		old pop.		t-test
	mean	CI	mean	CI	mean difference
F_{ST}	0.030	0.029-0.032	0.035	0.033-0.037	-0.0048**
G'_{ST}	0.048	0.043-0.053	0.069	0.062-0.076	-0.022***
Jost's D	0.031	0.028-0.035	0.047	0.042-0.052	-0.016***
Geo. Dist. (km)	4.26	3.71-4.83	4.42	3.84-5.01	-0.16

Figure 4.4. Isolation by distance graph. Correlation between Pairwise G'_{ST} values and logarithmic transformed geographic distance for all populations (all circles, continuous line (R_M = 0.17, β = 0.012, *P* = 0.001)), old populations (black circle, dashed line (R_M = 0.19, β = 0.018, *P* = 0.043)) and recent populations (open circle, no regression line (R_M = 0.069, β = 0.0044, *P* = 0.25)).

4.4.3 OVERALL GENETIC STRUCTURE

No significant clustering of the sampled populations was found using the admixture model in STRUCTURE. Individuals from the different populations were randomly assigned to one of the inferred groups (1 to K) at all values of *K* between 2 and 16. Using the admixture model with the LOCPRIOR option we observed a true value of 4 for *K*, as indicated by a maximum value of ΔK at this value. We observed a geographical clustering of three of these four genetic groups, with group 1 mainly restricted to populations in the east of the study area, and groups 3 and 4 mainly restricted to the west of the study area (Fig. 4.5).

Plants of individual recent populations were assigned to a mean of 6.1 old populations based on Geneclass2, indicating high gene flow between the different populations (Table 4.4). Individuals were more frequently assigned to nearby old populations than to more distant ones, as demonstrated by the significant negative correlation between the geographical distance between each recent population and the different source populations on the one hand, and the number of assigned plant individuals to each of these source populations on the other hand (β = -0.25, *P* = 0.018) (Fig. 4.6). We found a mean value of 0.22 for φ_p over all populations (Table 4.4).

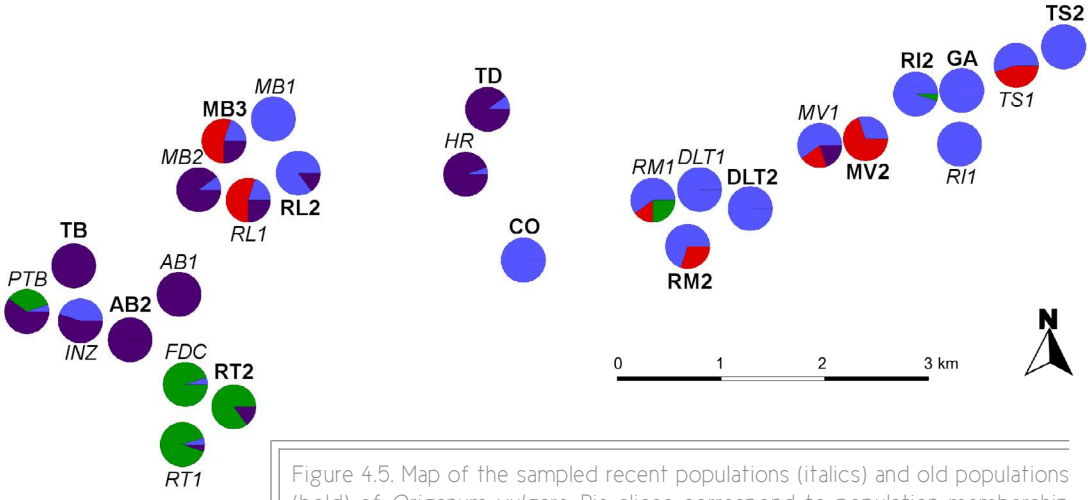


Figure 4.5. Map of the sampled recent populations (italics) and old populations (bold) of *Origanum vulgare*. Pie slices correspond to population membership to the four genetic groups defined by the Bayesian assignment analysis of Structure. Group 1: blue, group 2: red, group 3: green, group 4: purple. Population codes correspond to those in table 4.1.

Table 4.4. Assignment of individuals of recent populations (columns) to old populations (rows).

source ↓	AB1	DLT1	FDC	HR	INZ	MB1	MB2	MV1	PTB	RI1	RL1	RM1	RT1	TS1
AB2	4			1			1	2	1	1	2	1	2	4
CO	1	10	11	4	3	5	2	4	4	7	2	5	5	5
DLT2			1			1								
GA	1	2	1		1		2		2				2	
MB3				2	2	2	2	2	1		7	2		2
MV2	1							1						
RI2					1		1	1					1	
RL2	1												1	
RM2							1							
RT2			1				1				2			
TB	7	5	2	10	9	9	2	6	4	4	3	6	4	3
TD	4	3	4	3	3	3	8	4	7	8	4	6	5	6
TS2	1				1				1					
φ_p	0.17	0.31	0.32	0.29	0.23	0.26	0.17	0.15	0.18	0.29	0.17	0.22	0.15	0.18

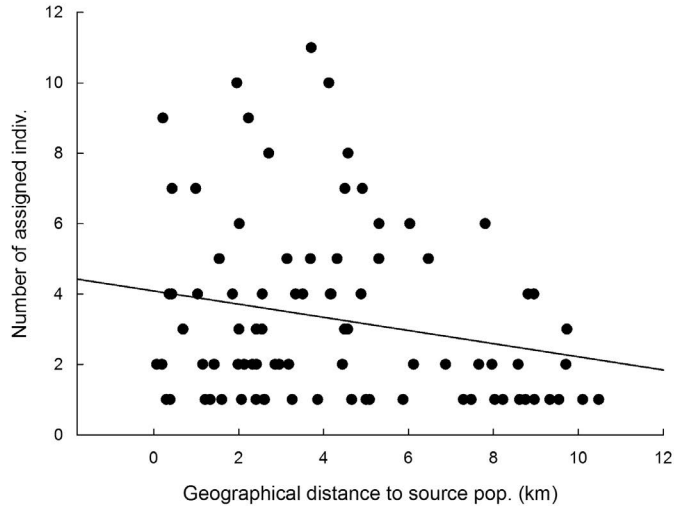


Figure 4.6. Spatial effect on genetic assignment. Correlation between the number of individuals of recent populations assigned to a source (old) population and the distance of the recent population to this source population (β = −0.25, P = 0.018). Data points represent assignment data from all 14 recent populations (Table 4.4).

4.5 DISCUSSION

The restoration of large patches of calcareous grassland following tree removal led to fast colonization of *Origanum vulgare*, resulting in several new populations in less than 10 years time. Contrary to our initial predictions, the percentage of female plants was not related to population age or population size, suggesting that no sampling effects had occurred in the sex structure of the founder populations after colonization (Nilsson & Ågren 2006). This is in contrast with recently founded populations of the gynodioecious *Beta vulgaris* ssp. *maritima*, which showed a higher variability in sex structure compared to older populations (De Cauwer *et al.* 2012). However, in this study, the increase in the percentage of female plants led to an increase in the number of alleles for large populations. This could possibly be explained by reproductive differences between hermaphroditic and female plants (the 'female advantage'). Since female plants are obligatory outcrossing, a higher number of female plants would increase outcrossing and thus led to higher genetic diversity (Dufay & Billard 2012). Linkage disequilibrium was also found to increase with decreasing population size in old populations, suggesting the occurrence of genetic drift in these populations, further confirmed by the occurrence of significant isolation by distance. This concurs with previous research on the effects of genetic drift on linkage disequilibrium, where populations with high spatial isolation and small population size were found to have higher levels of linkage disequilibrium (Tero *et al.* 2003; Zartman *et al.* 2006; Honnay *et al.* 2009).

The individuals of the newly established populations were assigned to on average 6.1 different source populations, suggesting considerable gene flow within our study area. This was confirmed by the lack of genetic differentiation between old and recent populations. Nevertheless, we observed a significant effect of the spatial configuration of the grasslands on gene flow, with gene flow into the recent populations mainly originating from nearby source populations. This was also reflected in the spatial aggregation of three of the four genetic groups defined by STRUCTURE. These results are in accordance with the findings of Lian *et al.* (2003), who observed that colonization of *Salix reinii* on Mount Fuji mainly consisted of seed recruitment of nearby populations supplemented by only limited seed recruitment over long distances. The diverse genetic origin of the individuals of recent populations in our study system

(φ_p = 0.22) suggests that it more likely follows the 'migrant pool' model according to Slatkin (1977), rather than the 'propagule pool' model. Significant isolation by distance was observed among old population (where colonization is lacking), but was absent among the recent populations (where colonization was occurring). Thus, we may assume that 'background' migration, mainly through pollen flow, is limited compared to seed flow at colonization in our system, also suggesting the occurrence of the 'migrant pool' model (Giles & Goudet 1997). This model predicts the absence of strong founder effects with respect to both genetic differentiation and genetic diversity in recent populations. Since the genetic composition of the source populations can have disproportionate effects on the genetic diversity of founder populations, the occurrence of founder effects can be expected to be even less likely for populations of species that exhibit low overall genetic differentiation, as is the case in our study system (F_{ST} = 0.040) (Whitlock & McCauley 1990, Yang *et al.* 2008).

Our results indeed showed that recent populations were not more genetically differentiated from each other than old populations. This is in accordance with previous research on the colonization of plant species that are characterized by high levels of gene flow and low overall genetic differentiation. Erickson *et al.* (2004), for example, observed comparable levels of genetic differentiation among old and new populations of *Myrica cerifera* during range expansion on Hog Island off the coast of Virginia (USA). Similar results during primary succession were found after colonization of glacier forelands by *Geum reptans* (Pluess & Stöcklin 2004) and *Saxifraga aizoides* (Raffl *et al.* 2006) in the European Alps, and by *Vaccinium membranaceum* at Mount St Helens in Washington (Yang *et al.* 2008). Colonization of recent lava flows by *Antirhea borbonica* on the Piton de la Fournaise volcano on La Réunion (Litrico *et al.* 2005) and by *Nassauvia lagascae var. lanata* on the Lonquimay volcano in Chile (López *et al.* 2010) also showed similar results. Antrobus & Lack (Antrobus & Lack 1993) observed equal levels of genetic differentiation among recent and old populations of *Primula veris* during secondary succession in young 'grassland' fragments in the Oxford region (UK). The high gene flow in our study can partly be explained by the relatively small distances among populations, taking into account that several populations also occur outside of the calcareous grassland fragments.

Whereas recent populations of *O. vulgare* were found to be little differentiated, old populations showed a higher degree of genetic differentiation. Furthermore, we observed significant isolation by distance for old populations, but not for recent populations. This suggests that old populations are moving towards a migration-drift

equilibrium. These populations likely became smaller and relatively more isolated when the grassland area decreased and landscape matrix quality decreased in the years prior to restoration (Adriaens *et al.* 2006). This could have led to decreased gene flow and increased genetic drift, in turn increasing genetic differentiation. This difference in the extent of among population gene flow (migration) and gene flow during colonization can be expected when seed flow is high throughout the system, but when seedlings experience high levels of inter- or intraspecific competition in old populations, leading to density-dependent mortality among migrants. Within recent founder populations, this mortality can be reduced or even absent due to lower levels of competition (Yang *et al.* 2008). In this case we can observe high gene flow towards recent populations due to seed flow, even when among population migration, mainly due to pollen flow is limited. In several studies, higher genetic differentiation or significant isolation by distance among old populations compared to recent populations has been attributed to lower gene flow, caused by higher geographical distance (Giles & Goudet 1997; Tremetsberger *et al.* 2003; Jacquemyn *et al.* 2006), historical levels of gene flow (Jacquemyn *et al.* 2004), or differences in population size and genetic drift (Vandepitte *et al.* 2007).

As predicted by the 'migrant pool' model of Slatkin (1977), no strong founder effects with respect to genetic diversity, and no evidence of recent bottlenecks in any of the recent populations was found. The number of alleles, expected heterozygosity and linkage disequilibrium of recent populations were also not significantly different from those of old populations. This is in accordance with several other studies of colonization in species with high levels of gene flow. Similar levels of genetic diversity for old and recent populations have been observed during both primary (Erickson *et al.* 2004; Pluess & Stöcklin 2004; Raffl *et al.* 2006; Esfeld *et al.* 2008; Raffl *et al.* 2008) and secondary (Antrobus & Lack 1993; Travis *et al.* 2002) succession. However, higher genetic diversity in recent populations, compared to old populations has also been observed in some instances (Tremetsberger *et al.* 2003; Yang *et al.* 2008). These authors argued that this difference can be caused by high rates of population growth after the occurrence of only a weak founder effect at colonization, since the population growth rate is known to affect the severity of genetic founder effects or bottlenecks (Nei *et al.* 1975; Tremetsberger *et al.* 2003; Green *et al.* 2012). Since many of the recent populations in our study system have become relatively large (mean population size of 1267 plants) within a short time period (<10 years), this can also explain the absence of strong founder effects.

Observed heterozygosity, on the other hand, was significantly lower in recent populations of *O. vulgare*, resulting in a higher inbreeding coefficient in these populations. The mean F_{IS} value of 0.083 for recent populations was low, however, and together with the absence of fitness effects on germination rate or seed weight in these recent populations, this suggests that inbreeding depression is absent. We can expect that future gene flow and population expansion of the recent populations will probably lead to a decreasing F_{IS} (Green *et al.* 2012).

Finally, the absence of pronounced founder effects in this study may also partly be explained by the occurrence of seed dispersal through time (Raffl *et al.* 2008). *Origanum vulgare* is known to form a persistent seed bank in our study area (Bossuyt & Honnay 2008b). All restored grasslands were historically grasslands before their afforestation and degradation. Therefore, it is not impossible that some seeds of *O. vulgare* were still present within the soil seed bank at the time of restoration. Recruitment from these seeds could have influenced the observed levels of gene flow (cf. Honnay *et al.* 2009). In this sense, gene flow towards the recent founder populations can be seen as the sum of temporal gene flow through the germination of dormant seeds, and spatial gene flow. Additionally, as stated before, pollen flow may also have occurred between the old and recent populations. Further analyses based on the assignment of seeds and seedlings to parent plant should be used to estimate the relative rates of pollen- and seed flow in our study system (Jones & Ardren 2003).

This study demonstrated that spontaneous plant colonization after habitat restoration can lead to new and viable populations, overcoming potentially important genetic founder effects, when several source populations are nearby. It has been suggested that the severity of founder effects is largely dependent upon the characteristics of the study area and the traits of the focal species, through mediating gene flow and population growth rate after colonization (Austerlitz *et al.* 2000; Tremetsberger *et al.* 2003; Sork & Smouse 2006; Green *et al.* 2012). This likely explains the absence of any founder effects in many studies. The rapid buildup of genetic diversity in restored populations, combined with low among population differentiation, can be expected to contribute positively to the overall viability of the *O. vulgare* metapopulation and also to mitigate the consequences of the genetic drift observed in the original source populations.

CHAPTER 5.

COMMUNITY (DIS)ASSEMBLY IN THE SOIL

Changes in the species and the functional trait composition of the seed bank during semi-natural grassland restoration: seed bank disassembly or ecological palimpsest?

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Helsen K, Hermy M, Honnay O. Changes in the species and the functional trait composition of the seed bank during semi-natural grassland assembly: seed bank disassembly or ecological palimpsest?

5.1 SUMMARY

Unlike above-ground plant community assembly, the processes that govern the assembly of the soil seed bank following severe habitat disturbance are poorly understood. Two hypotheses have been put forward in this context: (i) The 'ecological palimpsest hypothesis' assumes an accumulation of species in the seed bank through time; and (ii) the 'community disassembly hypothesis' assumes a gradual deterministic loss of species from the seed bank. Here, we investigated which hypothesis is applicable to the seed bank assembly of semi-natural grasslands, following forest clearance. Furthermore, we asked whether seed bank community divergence occurred at the species and at the functional trait level; and how seed bank composition was affected by the spatial configuration of the grassland patches. For this reason we performed a germination experiment to obtain the species composition of 106 seed bank samples, originating from seven grassland fragments and three restoration age classes. Community weighted means (CWM) were calculated for 26 functional traits. We evaluated changes in species and functional trait composition with increasing grassland age. Differentiation in species and trait composition was compared between age groups. The effect of spatial configuration of the grassland patches on pairwise species and trait differentiation was furthermore evaluated. Finally, we tested for the occurrence of nestedness of the seed bank communities through time. Our results showed that species richness of the seed bank decreased through time, which was reflected at the trait level by a replacement of traits associated with generalist therophytes by traits typical for chamaephytes and grassland specialists. Whereas species differentiation remained relatively constant, trait differentiation decreased through time. Only the species composition of ancient grasslands was affected by spatial configuration of the grassland patches. The seed bank composition of ancient grasslands was a nested subset of that of young grasslands. Our results suggest that the 'community disassembly hypothesis' is applicable to the temporal community change of the soil seed bank of semi-natural grasslands. Directly following forest clearance, a diverse seed bank is formed, followed by a gradual net loss of species. Although theory predicts this species loss to be driven by seed persistence traits, we found that this was not the case in our system, with species loss likely governed by functional changes in the above ground community. This disassembly process results in one deterministic end state at the trait level, but not at the species level.

5.2 INTRODUCTION

In an attempt to halt species loss and community degradation, large scale habitat restoration projects have been set up worldwide (Rice & Emery 2003; Rey Benayas *et al.* 2009). The success of these projects is largely dependent on the adequate understanding of the ecological processes that are playing during habitat restoration. In contrast to the considerable quantity of research performed on the above-ground plant community assembly following ecological restoration, relatively little attention has been devoted to processes affecting the within and among site variation of the species diversity of the soil seed bank following restoration. Most research so far has mainly focused on quantifying changes in seed bank species richness and similarity between seed bank and standing vegetation composition, but often lacks to generate general seed bank assembly patterns (e.g. von Blanckenhagen & Poschlod 2005; Bisteau & Mahy 2005; Koch *et al.* 2011). Yet, clear insight in seed bank assembly patterns is of considerable relevance for both restoration and conservation, since a well-developed seed bank may act as a genetic and taxonomic reservoir for the present plant community, buffering populations against environmental disturbance and stochasticity (Kalamees & Zobel 2002; Honnay *et al.* 2008; Mandák *et al.* 2012). Furthermore, although dependent upon the habitat type, seed banks can aid the restoration of the above-ground community (Bossuyt & Honnay 2008a).

Two non-exclusive hypotheses can be put forward regarding changes in species composition of the seed bank following a severe disturbance event, which usually accompanies initial restoration measures. The first hypothesis states that the species richness of the seed bank will gradually increase, with viable seeds of species of each successional stage accumulating in the soil, resulting in an 'ecological palimpsest' end state composition (Davies & Waite 1998). Following this hypothesis, the seed bank composition of early restoration sites is predicted to be a nested subset of the seed bank composition of the end state community (Davies & Waite 1998). Some evidence for this hypothesis has been obtained during grassland succession following grazing and mowing abandonment (Davies & Waite 1998; Falinska 1999). A second hypothesis assumes that the progressing aboveground assembly is accompanied by a belowground community disassembly of the seed bank. According to this hypothesis, the seed bank is formed immediately following a disturbance event, after which species are gradually and non-randomly lost, depending on their seed characteristics (Zavaleta *et al.* 2009).

According to this hypothesis the seed bank composition of the end state community is predicted to be a nested subset of the seed bank composition of early restoration sites (Royo & Ristau 2012). Several studies focusing on changes in forest seed bank composition following a disturbance event have found partial support for this hypothesis (van Calster *et al.* 2008; Plue *et al.* 2010; Royo & Ristau 2012), but evidence for other vegetation types is currently lacking (Bekker *et al.* 2000; but see Kalamees & Zobel 1997).

At the functional trait level, however, above-ground community assembly has been observed to remain more or less deterministic, resulting in strong trait convergence among different restoration sites in time. This convergence is thought to be solely defined by the available niches, which are in turn defined by site-level abiotic and climatic conditions (Fox 1987; Fukami *et al.* 2005; Petermann *et al.* 2010). When assuming that seed bank patterns are mainly defined by the present (and former) above-ground community, changes in seed bank composition can also be expected to be deterministic at the functional trait level. This can in turn result in a strong functional signal during seed bank assembly (Meers *et al.* 2012). Seed bank trait composition can alternatively be expected to be governed by functional traits that affect seed longevity, also leading to a similar suit of traits present among different restoration sites (Falinska 1999; Fagan *et al.* 2010; Pakeman & Eastwood 2013). The limited research performed so far on seed bank functional trait composition has indeed observed directional changes during assembly, leading to an increase in stress-tolerant species, mean plant longevity and fruit dispersal, and a decrease in the number of therophytes/ruderal species, associated with a decrease in mean flowering duration and nutrient preference (Ellenberg N) for grassland assembly (Bossuyt *et al.* 2006; Fagan *et al.* 2010). Research in forests showed a temporal increase in mean seed longevity and a decrease in mean seed weight of the seed bank since the last disturbance (van Calster *et al.* 2008).

In this chapter we investigated changes in the seed bank plant community of semi-natural grasslands at both the species and the functional trait level, following grassland restoration on former forest stands. Grassland communities were sampled in seven separate grassland fragments over three age groups using a high number of seed samples for each location (80 point samples per hectare grassland). More specifically, we tried to answer the following questions:

1. Does species diversity and composition of the seed bank of restored grasslands change with progressing assembly of the standing vegetation, and how is this reflected at the functional trait level?

2. Are changes in seed bank composition deterministic at the species and the functional trait level? More specifically, does differentiation among seed bank communities decrease with increasing time since restoration?

3. Is the seed bank composition at the species and the trait level affected by the spatial configuration of the grassland patches?

4. Can we find support for either the 'ecological palimpsest' or the 'community disassembly' hypotheses? Or more specifically, how are the different seed bank communities nested in each other with respect to time since restoration?

5.3 MATERIALS AND METHODS

5.3.1 DATA COLLECTION

Seed bank samples were collected from seven calcareous grassland fragments over three age groups; young (restored between 2005 and 2007; 5 till 7 years old), middle-aged (restored between 2001 and 2004) and old grassland patches (ancient calcareous grassland), during September of 2012 (Table 5.1, Fig. 5.1). At each site two 2 x 2 m plots were randomly selected for each hectare of grassland, resulting in a total of 106 plots. As recent research indicated that the low number of soil samples in many seed bank studies lead to considerable bias in the observed diversity and composition patterns of the seed bank community (Plue & Hermy 2012), we collected a high number of soil samples for each location. At each plot 40 random soil samples were taken with a 2 cm diameter soil auger and bulked together. Due to the shallow nature of the soil, samples were taken up to the mineral bedrock (<15 cm). The top cm of each sample was removed to exclude transient seeds present at the surface. Samples were stored in paper bags in a cool and dark environment until further processing. The samples were concentrated, using the methodology of Ter Heerdt *et al.* (1996), using a coarse (4 mm) and a fine (0.2 mm) sieve in order to remove both coarse and fine soil material and vegetation parts. The concentrated samples were spread out on plastic containers (40 x 45 cm) filled with sterile potting soil. Seeds in these samples were allowed to germinate under a light regime of 16h light and 8h darkness and day temperatures

ranging between 20 and 30°C. The plastic containers were watered by capillarity and contained a thin layer of lava gravel at the bottom to prevent water logging. Five control containers without seed samples were included in the experiment to test for contamination of airborne seeds or seeds present in the potting soil. After 15 weeks of germination the samples were subjected to a cold stratification period (2°C) for eight weeks. Following cold stratifications the samples were subjected to a second germination period of 15 weeks. All emerging seedlings were counted and removed, with unidentified seedlings transplanted for later identification.

5.3.2 PLANT TRAITS

26 functional plant traits were selected for further analyses, based upon their relevance for community assembly, including the processes of dispersal, establishment and persistence (cf. Weiher *et al.* 1999, Appendix 2.2). Trait values were extracted from different sources (Fitter & Peat 1994; Lambinon *et al.* 1998; Klotz *et al.* 2002; Poschlod *et al.* 2003; Kleyer *et al.* 2008). The Seed longevity index was calculated according to Bekker *et al.* (1998), based on seed longevity data of Thompson *et al.* (1997). In total, 92% of all trait values were available for all species. Functional traits: plant height, seed mass and seed number were logarithmically transformed before further analysis, to reduce the effect of extreme values.

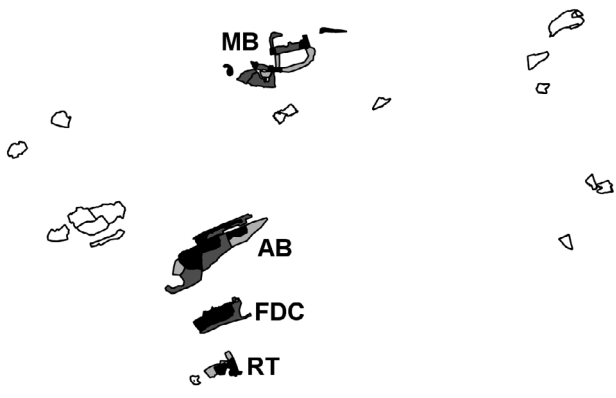
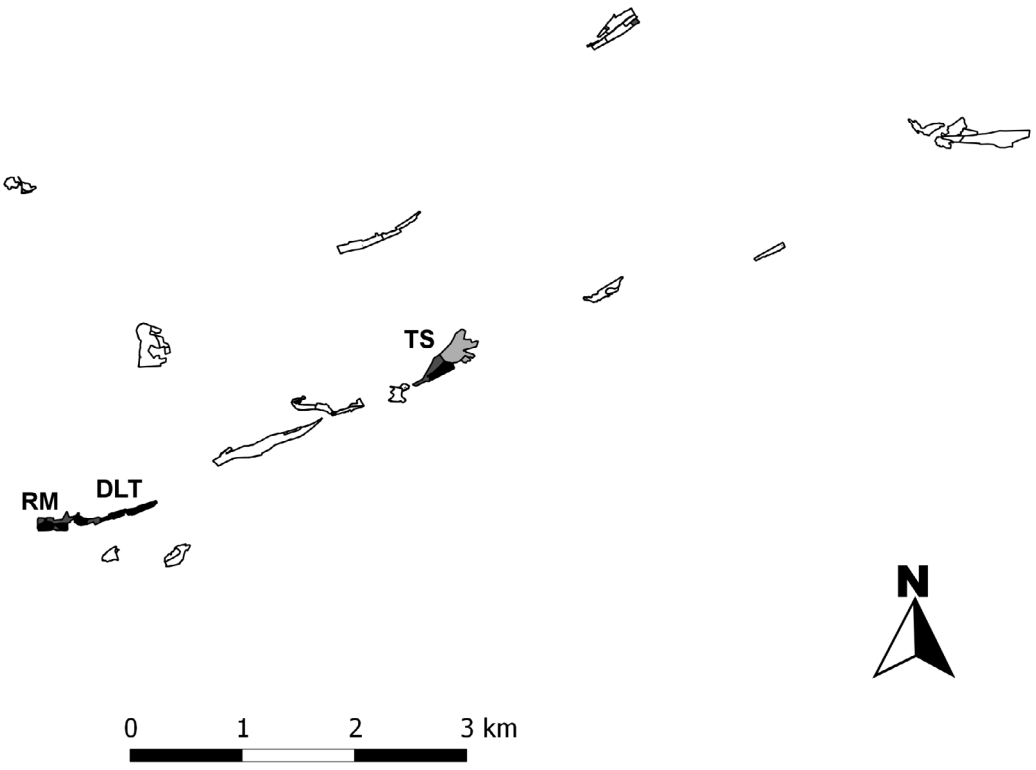


Figure 5.1. Sampled young (light grey), middle-aged (dark grey) and old (black) grassland patches in the Viroin valley. Grassland codes: AB: Les Abannets; DLT: Desous le Transoi; FDC: Fondry des Chiens; MB: Montagne-aux-Buis; RM: Roche Madoux; RT: Roche Trouée; TS: Tienne Saumières.

The weighted average value of each trait *t* was calculated for each plot *j* (community-weighted trait means, CWM), according to the following formula: $CWM_t = \sum_{i=1}^{S} p_{ij} t_i$. In this formula, p_{ij} is the square root transformed number of seeds of species *i* in plot *j* and *S* is the total number of species in that plot (Díaz *et al.* 2007). Prior to CWM calculation, we recoded nominal traits as dummy variables and standerdized trait state values of each ordinal and ratio trait to range from zero (minimum) to one (maximum) over all species of the species pool. This guarantees equal weight for all traits during subsequent analyses. CWM values for all 26 traits were used for the construction of a *plot x trait* matrix. CWM calculations were performed in FDiversity (Casanoves *et al.* 2011).



	Young grassland patches (5–7 y.o.)					Middle-aged grassland patches (8–11 y.o.)					Old grassland patches (ancient)				
Grassland ID	# plots	Dens.	S_{tot}	S_{gen}	S_{spec}	# plots	Dens.	S_{tot}	S_{gen}	S_{spec}	# plots	Dens.	S_{tot}	S_{gen}	S_{spec}
Les Abannets	7	2285.0 (±1780.4)	10.7 (±4.4)	8.3 (±4.1)	2.4 (±1.1)	19	2613.5 (±1148.7)	12.7 (±4.1)	9.8 (±3.8)	2.9 (±1.4)	7	1830.3 (±519.8)	11.1 (±3.6)	4.9 (±3.3)	6.3 (±2.0)
Desous le Transoi	0	-	-	-	-	2	1512.0 (±1125.4)	8.5 (±4.9)	5.5 (±2.1)	3.0 (±2.8)	5	2403.2 (±)1054.8	9.6 (±1.9)	5.0 (±1.6)	4.6 (±1.9)
Fondry des Chiens	0	-	-	-	-	7	2773.8 (±2544.6)	10.9 (±2.6)	7.6 (±2.6)	3.0 (±1.9)	7	1989.4 (±953.8)	9.7 (±2.4)	3.6 (±2.1)	6.1 (±2.6)
Montagne-aux-Buis	5	6016.0 (±4034.0)	16.2 (±4.0)	12.4 (±1.9)	3.8 (±2.8)	9	6746.4 (±3399.7)	11.3 (±3.6)	5.2 (±3.9)	6.0 (±2.6)	8	2725.5 (±1816.5)	9.6 (±3.6)	3.4 (±2.3)	6.3 (±2.1)
Roche Madoux	0	-	-	-	-	4	2287.8 (±610.8)	9.3 (±1.7)	5.0 (±0.8)	4.3 (±1.7)	3	1989.4 (±286.9)	12.0 (±1.7)	7.7 (±1.2)	4.3 (±0.6)
Roche Trouée	3	1299.8 (±1059.7)	7.7 (±4.5)	6.3 (±4.2)	1.3 (±1.5)	0	-	-	-	-	3	2997.4 (±2422.0)	9.3 (±3.1)	5.3 (±1.2)	4.0 (±2.0)
Tienne Saumières	9	3598.6 (±2847.8)	14.7 (±6.1)	11.1 (±4.8)	3.6 (±1.9)	5	5029.3 (±3990.5)	13.0 (±7.4)	8.2 (±5.8)	4.8 (±2.6)	3	4986.8 (±4263.4)	12.3 (±1.2)	4.7 (±2.5)	7.7 (±1.5)
Total	24	3431.8 (±3005.2)	13.0 (±5.6)	10.0 (±4.3)	3.0 (±2.0)	46	3622.5 (±2834.0)	11.7 (±4.2)	7.8 (±4.0)	3.9 (±2.3)	36	2513.1 (±1759.6)	10.3 (±2.8)	4.6 (±2.4)	5.8 (±2.1)

5.3.3 DATA ANALYSIS

The species richness (S) and the evenness (E) of the soil seed bank were calculated for each plot, including all species, generalist species only and specialist species only. Total germinated seed density per m^2 was also calculated including all species and for generalist species and specialist species separately. Specialist species were defined as species mainly confined to calcareous grasslands in Belgium (Lambinon *et al.* 1998; Van Landuyt *et al.* 2006). Each plot was assigned to one of the three earlier defined grassland patch age classes (young, middle-aged and old). Seed bank species richness and seed density were compared between the different age classes using linear mixed models (LMM, *SPSS Statistics 20.0*). Since plots were clustered in seven grasslands, grassland identity was included as a random factor in these models. Semi-partial R^2_β coefficients were calculated using the method of Edwards *et al.* (2008). Pairwise comparisons of the three age classes were corrected for increased type-I error by applying Bonferroni corrections on the resulting p-values. Species richness and seed density of specialist species and generalist species was divided by the total species richness/seed density of each plot, respectively, to obtain the proportional richness/seed density before analysis with LMM. All three measures of evenness were squared and all three measures of seed density were square root transformed to obtain a normal distribution before analysis. Seed bank longevity was compared between generalist and specialist species using a Mann-Whitney U test. Variation in species composition of the seed bank between the different age classes were visualized using Detrended Correspondence Analysis (DCA) on the square root transformed *plot x species* matrix, performed in PC-ORD 6.0 (McCune & Mefford 1999). Significant differences in species composition between age classes were inferred using the Multi-Response Permutation Procedure (MRPP) based on Bray-Curtis distances in species composition among plots, calculated on the square root transformed *plot x species* matrix in PC-ORD 6.0 (McCune & Mefford 1999). MRPP is a nonparametric permutation method used for testing multivariate differences among pre-defined groups (McCune & Grace 2002). Pairwise comparison of differences in species composition between age classes was

Table 5.1. Overview of sampled grasslands for the three age groups separately. Means and standard deviations given. Dens.: number of germinated seeds per m^2 (seed density); S: mean species richness; gen: generalist; spec: specialist; y.o.:years old.

also performed with MRPP, with application of Bonferroni corrections of the resulting p-values. Since the number of plots was not equal among grasslands, we were not able to include grassland identity as a nested factor in the design, to correct of the dependent nature of the data.

A Principal Component Analysis (PCA) was performed on the *plot x trait* matrix to visualize differences in the functional trait composition of the seed bank between age classes in PC-ORD 6.0 (McCune & Mefford 1999). Significant differences in trait composition of seed bank communities between age classes was tested using SumF in PC-ORD 6.0 (McCune & Mefford 1999). This method calculates an *F* statistic for each functional trait (CWM) independently, based on the difference between age classes. To test for significant difference in overall trait composition of the seed bank between age classes, these univariate *F* statistics were summed and compared to the distribution of *F* statistics based on 9999 randomizations of the data under the null hypothesis of no difference. Pairwise comparison of age classes were also included in the SumF analysis. We were not able to include grassland identity as a nested factor in the design, because the number of plots was not equal among grasslands. To correct for the dependent nature of our data we also performed independent LMM on the CWM's for each trait, with grassland patch age as a fixed factor and grassland identity as a random factor. Significance levels were inferred using a bootstrapping procedure of 9999 bootstraps to correct for the increased type-I error accompanied with multiple statistic testing (*SPSS Statistics 20.0)*. The results of these LMMs were compared to those of the SumF analysis to infer the effects of the data clustering on our results.

Pairwise differentiation in species composition between seed bank plots was characterized using three differentiation measures based on the square root transformed *plot x species* matrix; the Bray-Curtis dissimilarity (D_{BCS}), Raup-Crick beta-diversity (β_{RC}) and a community analogue of the fixation index in population genetics (F_{ST}). β_{RC} is a presence-absence based probabilistic measure of differentiation, that is independent of species richness (Raup & Crick 1979). D_{BCS} and F_{ST} are both based on abundance data, with F_{ST} being the difference in relative cover of species between plots, calculated as defined by Vellend *et al.* (2004) (Baeten *et al.* 2010). Since F_{ST} is known to be strongly affected by dominant species (alleles) (Meirmans & Hedrick 2011), F_{ST} was calculated leaving out the five most frequent species (occurring in >43% of all plots). Pairwise differentiation in trait (CWM) composition between seed bank plots was characterized using two differentiation measures; the Euclidean distance (D_E) and the Bray-Curtis dissimilarity (D_{BCT}).

Species and trait differentiation among plots were compared between age classes using ANOVA analysis on pairwise species and trait differentiation values. Since plots are clustered within seven grasslands, mean pairwise species and trait differentiations were first pooled for each combination of grasslands for this analysis, resulting in a *grassland x grassland* differentiation matrix. Because of the dependency of pairwise data, a bootstrapping procedure of 9999 bootstraps was applied for the calculation of the test statistics and 95% confidence intervals (SPSS Statistics 20.0).

The effect of geographic distance on the species and trait differentiation of the seed bank plots (isolation by distance) was tested for all plots and for plots of the three age classes (young, middle-aged and old) separately, by regressing pairwise species and trait differentiation (D_{BCS}, F_{ST}, β_{RC}, D_E and D_{BCT}) on pairwise logarithmically transformed spatial distances using partial Mantel tests in PC-ORD 6.0 (McCune & Mefford 1999). To correct for the clustered nature of the plots in seven grasslands, we partialled out the effect of a third matrix containing grassland identity of the plots. A total of 9999 random permutations were performed. Geographical distances were calculated as the Euclidean distance between seed bank plots using QGIS 1.8.0 (Quantum GIS Development Team 2010).

Finally, we performed a nestedness analysis using the 'nestedness metric based on overlap and decreasing fill' (NODF) to test for nested seed bank patterns within the maximally packed *plot x species* matrix. For this analysis we used both a presence/absence and an abundance based NODF null model, randomized with fixed total abundance. Significance was tested using 1000 randomisations (Almeida-Neto *et al.* 2008; Almeida-Neto & Ulrich 2010). If significant nestedness was observed, we tested whether this pattern is related to grassland patch age. This was accomplished by comparing the mean rank-order of the plots in the maximally packed matrix between different age classes using a LMM analogous to those performed on the species diversity measures (*SPSS Statistics 20.0)*. This procedure allows us to evaluate if the species composition of ancient seed bank plots is a subset of that of restored seed bank plots.

5.4 RESULTS

5.4.1 SPECIES AND TRAIT DIVERSITY

A total of 4266 seeds germinated, of which 1035 seeds originated from young grassland patches, 2094 seeds from middle-aged grassland patches and 1137 seeds from old (ancient) grassland patches. These figures correspond to a mean seed density of 43.1 seeds per young grassland plot (3431.8 seeds/m^2), 45.5 seeds per middle-aged grassland plot (3622.5 seeds/m^2) and 31.6 seeds per old grassland plot (2513.3 seeds/m^2) (Table 5.1). This can be translated to the species level as a total of 87 species germinating in young grasslands plots, 114 species in middle-aged grassland plots and 80 species germinating in old grassland plots, resulting in a total of 140 observed seed bank species (Appendix 5.1). Two species (*Juncus tenuis* and *Salix caprea*) were observed in the control containers, indicating possible contamination of the sterile soil. Since these species were also observed in other containers, they were removed from the species list.

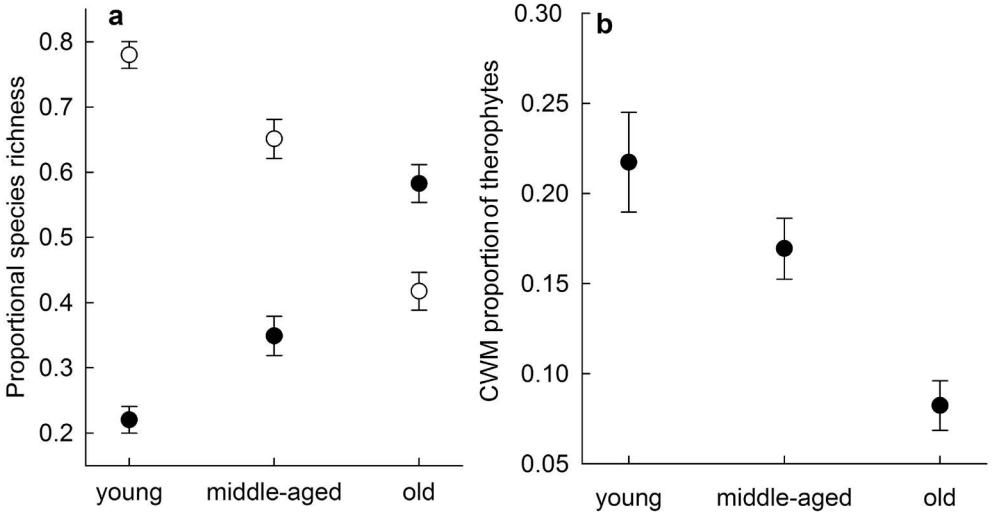


Figure 5.2 a. Changes in the proportional species richness with increasing seed bank age for generalist species (open circle) and specialist species (full circle) b. Changes in the community weighted mean proportion of therophytes with increasing seed bank age. Means and standard error bars are presented for each age class.

Total species richness was significantly higher for the seed bank of young grassland patches compared to old grassland patches (Table 5.2), a trend also visible in the total seed density, although not significant (F = 2.44, P = 0.092, Table 5.2). This same pattern was even more pronounced for the proportional generalist species richness and seed density, with a gradual decrease through time over the three age classes (Fig. 5.2a). In contrast, the number of specialist species gradually increased through time, resulting in the highest occurrence in the seed bank of old grassland patches, both with respect to the number of seeds and the number of species (Table 5.2, Fig. 5.2a). The evenness of specialist species was found to be significantly lower in old grassland patches compared to young grassland patches. All other measures of evenness were unaffected by grassland age (Table 5.2). The mean seed bank longevity was significantly higher for generalist species compared to specialist species (Z = 3.2, P = 0.0015, n = 126).

Table 5.2. Parameter estimates of the model relating diversity indices to grassland age using LMM. Test statistic and semi-partial R^2_β given for age (n = 106). β-coefficients (means) are given for each age group. Pairwise comparisons (contrasts) were generated after Bonferroni correction. S: species richness; Dens.: seed density; E: Evenness; m.a.: middle-aged (age group); Rank: rank-order of the plots in the maximally packed matrix. Significance: *0.05 ≥ P-value > 0.01 **0.01 ≥ P-value > 0.001 ***0.001 ≥ P-value.

	F	R^2_β	β old	β m.a.	β young	contrast old – m.a.	contrast m.a. – young	contrast old – young
S	3.2*	0.034	10.33	11.62	12.81	-1.30	-1.19	-2.48*
S spec.	34.0***	0.249	0.56	0.34	0.21	0.23***	0.13**	0.36***
S gen.	32.6***	0.241	0.44	0.66	0.79	-0.22***	-0.14**	-0.35***
√ (Dens.)	2.4	0.023	5.31	6.31	5.64	-1.00	0.68	-0.32
√ (Dens. spec.)	25.4***	0.198	0.74	0.54	0.40	0.21***	0.14*	0.35***
√ (Dens. gen.)	17.6***	0.146	0.63	0.79	0.90	-0.16***	-0.11*	-0.27***
E^2	3.1	0.029	0.84	0.80	0.86	0.044	-0.15	-0.19
E spec.^{2}	3.8*	0.036	0.64	0.68	0.83	-0.040	-0.15	-0.19*
E gen.^{2}	0.3	0.003	0.60	0.56	0.55	0.034	0.015	0.049
Rank	3.2*	0.032	12.67	11.37	10.21	1.30	1.16	2.46*

The DCA ordination of the species occurrence in the seed bank shows a clustering of plots originating from young grassland patches and plots originating from old grassland patches. Plots of middle-aged grassland patches on the other hand show a larger spread, suggesting a more diverse species composition (Fig. 5.3a). The MRPP analysis confirms the presence of a significant difference in species composition between seed bank plots of different grassland age (Table 5.3).

The PCA ordination of the community weighted mean trait composition (CWM) of the seed bank shows a clear clustering of plots originating from young grassland patches and old grassland patches, with a much wider range for plots originating from middle-aged grassland patches, largely equivalent to the patterns observed at the species level (Fig. 5.3b). The SumF analysis showed that a significant difference in overall trait composition occurs between seed bank plots from different age classes (Table 5.3). Bootstrapping LMM showed that these overall differences can be translated to clear shifts in independent functional traits between age groups (Appendix 5.2). The seed bank composition of old grassland patches was characterized by a lower proportion of phanerophytes and therophytes compared to middle-aged grassland patches, leading to a lower mean value for soil nutrient preference, seed shape, seed number, seed longevity and the proportion of selfing and autogamous species, and a higher mean value for life span and age of first flowering, all plant trait syndromes typically associated with therophytes (Fig. 5.2b). We also observed a significantly higher proportion of chamaephytes in old seed bank communities, in turn associated with a higher mean value for insect pollination. The seed bank of old grassland patches was furthermore characterized by a higher mean value of light preference, autochory and epizoochory

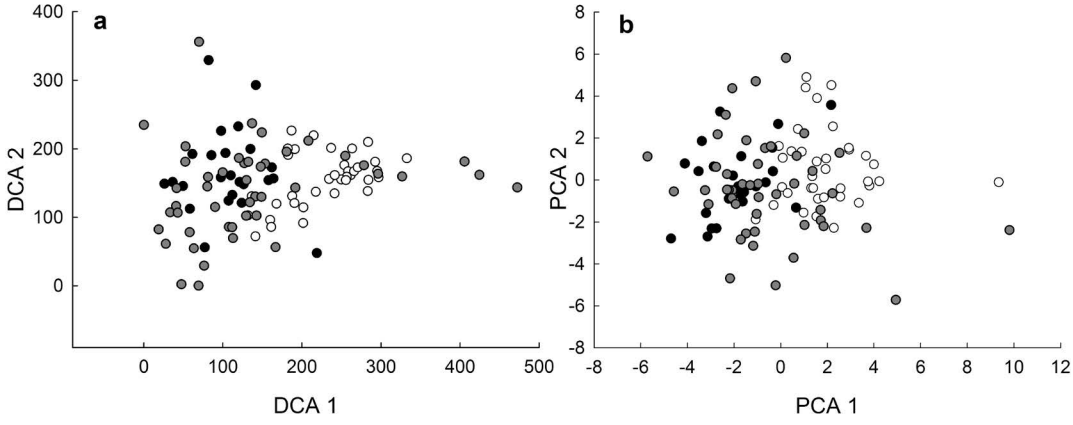


Table 5.3. Parameter estimates of performed MRPP and SumF analyses relating respectively species and trait (CWM) composition to grassland patch age. t-values are given for MRPP, F-values for SumF. SumF analysis was based on 9999 randomisation. m.a.: middle-aged (age group). Significance: *0.05 ≥ *P*-value > 0.01 **0.01 ≥ *P*-value > 0.001 ***0.001 ≥ *P*-value.

	overall	contrast old – m.a.	contrast m.a. – young	contrast old – young
Species composition (MRPP)	-16.41***	-14.48***	-2.23*	-16.84***
Trait composition (SumF)	218.96***	255.94***	63.97*	420.49***

and a lower mean value for leaf size, clonality (reproductive type) and hemerochory. Several of these changes in trait composition from middle-aged toward old seed bank communities were also visible in the transition from young toward middle-aged seed bank communities (Appendix 5.2). However, we also observed several independent trait differences between the seed bank of young and middle-aged grassland patches. The seed bank of young grassland patches was characterized by a higher mean value for dysochory and seed length and a lower proportion of rosette species compared to both middle-aged and old grassland patches. Mean seed mass, finally, was observed to increase from young toward middle-aged seed bank communities, followed by a decrease from middle-aged to old seed bank communities.

Figure 5.3. a. DCA ordination on the square root transformed *plot x species* matrix. b. PCA on the *plot x CWM* trait matrix. Separate symbols for seed bank plots of young grassland patches (full circle), middle-aged grassland patches (grey circle) and old grassland patches (open circle).

5.4.2 SPECIES AND TRAIT DIFFERENTIATION

Species differentiation among grasslands was significantly higher for middle-aged seed bank communities compared to both old and young seed bank communities based on F_{ST} (Table 5.4). No significant differences in species differentiation among age groups were observed for D_{BCS} and β_{RC}. Trait differentiation among grasslands showed a significantly lower differentiation for old seed bank communities compared to middle-aged seed bank communities, based on D_E and D_{BCT} (Table 5.4). The average geographic distance separating grasslands, however, was not significantly different between the three age classes (Table 5.4).

	contrast old – m.a.		contrast m.a. – young		contrast old – young	
	mean diff.	CI	mean diff.	CI	mean diff.	CI
species: D_{BCS}	0.015	-0.039 – 0.072	0.017	-0.035 – 0.066	0.032	-0.034 – 0.099
species: β_{RC}	-0.062	-0.146 – 0.025	0.040	-0.093 – 0.156	-0.022	-0.148 – 0.093
species: F_{ST}	-0.060*	-0.084 – -0.037	0.042*	0.005 – 0.081	-0.018	-0.051 – 0.017
traits: D_E	-0.167*	-0.236 – -0.098	0.084	-0.039 – 0.208	-0.083	-0.196 – 0.032
traits: D_{BCT}	-0.026*	-0.039 – -0.014	0.014	-0.008 – 0.036	-0.012	-0.008 – 0.032
geographic distance	-122.9	-1712.1 – 1663.6	-712.6	-4001.6 – 2699.8	-835.5	-4141.2 – 2651.1

5.4.3 SPATIAL EFFECTS

We observed a significant increase in species differentiation among seed bank plots with increasing geographical distance (isolation by distance) based on D_{BCS}, β_{RC} and F_{ST} for old seed bank communities, but not for young and middle-aged seed bank communities or for all plots together (Table 5.5, Fig. 5.4). At the trait level, we observed no significant isolation by distance for all plots, nor for plots of the different age classes separately, based on D_E and D_{BCT} (Table 5.5).

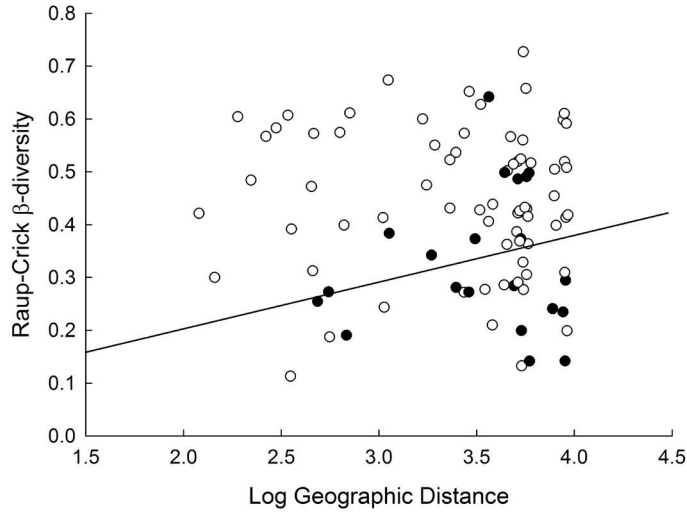


Figure 5.4. Correlation between pairwise Raup-Crick β-diversity and logarithmic transformed geographic distance for seed bank plots of old (ancient) grassland patches (black circles; continuous regression line; R_M = 0.125, P = 0.0085). Pairwise differences for other grassland patches are visualized by open circles. Graph was constructed using mean distances between grasslands.

Table 5.4. Parameter estimates of performed pairwise comparisons after bootstrapping ANOVA on pairwise differentiation and geographic distance between different grassland patch age classes. All tests are based on 9999 bootstraps. CI: 95% confidence intervals. Mean diff. = mean difference. m.a.: middle-aged (age group). Note that significance is not inferred from *P*-values but from the CI's, '*' only indicates significance, not the size of the *P*-value.

	all R_M	old R_M	m.a. R_M	young R_M
species: D_{BCS}	-0.049	0.13*	-0.013	-0.13
species: β_{RC}	-0.031	0.14**	-0.018	-0.11
species: F_{ST}	0.020	0.13*	0.12	-0.03
traits: D_E	-0.044	0.091	-0.0072	-0.084
traits: D_{BCT}	-0.049	0.094	-0.026	-0.12

Table 5.5. Parameter estimates of performed partial Mantel tests relating pairwise differentiation to logarithmically transformed pairwise geographic distance after correction of grassland identity. All tests are based on 9999 bootstraps. R_M: partial standardized Mantel regression coefficient. m.a.: middle-aged (age group). Significance: *0.05 ≥ *P*-value > 0.01 **0.01 ≥ *P*-value > 0.001 ***0.001 ≥ *P*-value.

5.4.4 NESTEDNESS

Seed bank composition showed a significantly nested pattern for both the presence-absence (NODF = 18.9, Z = -21.7, P < 0.001) and abundance based analysis (WNODF = 8.5, Z = -16.3, P < 0.001). This nested pattern was significantly affected by grassland age with a significant difference between young and old grassland patches (Table 5.2). Although not significant, middle-aged grassland patches showed a mean rank intermediate to that of young and old grassland patches, suggesting a gradual increase in rank number with increasing seed bank age. This indicated that the species composition of the seed bank of ancient grassland patches is, at least partly, a nested subset of the species composition of the seed bank of restored grassland patches.

5.5 DISCUSSION

5.5.1 SPECIES AND TRAIT DIVERSITY

Species richness in the soil seed bank is often observed to decrease during secondary succession, usually accompanied by a decline in seed density (e.g. Davies & Waite 1998; Falinska 1999; Bekker *et al.* 2000). Our study confirms this pattern during calcareous grassland community assembly following restoration. Although the total number of species per soil sample decreased through time, we observed that the proportional number of specialist species and its number of seeds gradually increased. This suggests a significantly greater loss of generalist species and seeds from the soil seed bank than an gain of specialist species. This pattern can be expected if specialist species are characterized by a longer seed longevity than generalist species (Royo & Risteau 2012). This, however, is not the case in our study system, where generalists from restored calcareous grasslands mainly consist of annual therophyte species (Bisteau & Mahy 2005; Fagan 2010; see also chapter 2), showing a significantly higher seed longevity than specialist species. The observed pattern can alternatively be explained by changes in seed input from the above-ground vegetation (Bekker *et al.* 2000; Bisteau & Mahy 2005). Previous research on these grasslands has indeed

established that generalist species are gradually replaced by specialist species in the above-ground community, with increasing time since restoration (see chapter 2). This suggests that the seed bank of generalist species is gradually depleted through predation and mortality. For specialist species on the other hand, these processes are likely mitigated by the influx of new seeds from the above-ground vegetation (Falinska 1999; Bekker *et al.* 2000; Rosef 2008). This shift in seed bank composition from a generalist towards a specialist dominated community was accompanied by a significant, directional change in the species composition of the soil seed bank communities as observed in the DCA and MRPP analyses. Although the results of the MRPP were not corrected for the clustering of the data in seven grassland fragments, we believe that these results would be little affected by this clustering. Nevertheless the results should be interpreted with caution.

More interestingly, this species composition shift is accompanied by strong changes in its functional trait composition. The observed functional seed bank shift consisted of a partial replacement of therophytes and phanerophytes by chamaephytes, consistent with the replacement of generalist by specialist species (see chapter 2). This is partly consistent with the study of Fagan *et al.* (2010), reporting the replacement of therophytes with hemicryptophytes in the seed bank, following initial restoration of calcareous grasslands in southern England. The changes in life form composition, observed in our study can help explain the directional changes in trait composition. The loss of therophytes, for example, can explain the changes in the mean seed size, number and longevity, nutrient preference and life span of the seed bank community (Fagan *et al.* 2010; Meers *et al.* 2012). Again, we can assume that most of these trait changes are driven by the progressing changes in the seed influx composition from the above-ground vegetation, shifting from a generalist dominated toward a specialist dominated community (cf. Klimkowska *et al.* 2010). When focussing specifically on seed traits, however, we observe patterns opposite to those predicted from theory and other observational studies (Falinska 1999; Van Calster *et al.* 2008; Royo & Risteau 2012). Indeed, following the 'community disassembly' framework, progressing assembly at the soil seed bank level is expected to be, at least partly, governed by the predictable loss of species producing only a small number of seeds and of species with large seeds or low seed longevity, all traits expected to reduce the survival of a species in the seed bank (Thompson *et al.* 1993; Bekker *et al.* 1998). Our results indicate, however, that the loss of therophytes from the above-ground vegetation has a stronger functional signal than the predictable trait governed loss of seeds from the seed bank. This is in accordance with a hayfield succession study in the Netherlands of Bekker *et al.* (2000), who also

observed a reduction in the mean seed longevity of the seed bank with progressing succession. Bossuyt *et al.* (2006) also observed the absence of a predictable loss of species from the seed bank based on seed bank longevity with progressing succession from calcareous grassland toward scrub in our study area. The seed dispersal capacity spectrum was also observed to significantly respond to time since restoration, with the replacement of hemerochore and dysochore species by autochore and epizoochore species. This possibly reflects the later arrival of the latter species in the community because of reduced dispersal capacities (see chapter 2). Interestingly, Van Calster *et al.* (2008) observed no effects of dispersal ability on seed bank assembly in temperate forests.

5.5.2 SPECIES AND TRAIT DIFFERENTIATION

Looking at species differentiation, only F_{ST} was observed to significantly differ among age groups. F_{ST} was higher for seed bank plots of middle-aged grassland patches compared to those of both old and young grassland patches. This indicates a higher community drift (divergence in local abundances) for middle-aged grasslands (Vellend *et al.* 2004; Baeten *et al.* 2010). This can possibly be explained by the heterogenous nature of this age group. The assembly rate within our study area has been found to be different among restoration sites, dependent upon their isolation, resulting in a variation in the advancement of assembly (see chapter 2). Since below-ground assembly is likely affected by above-ground assembly, we are inclined to believe that the below-ground assembly rate will also be affected by other factors, possibly explaining stronger differences among middle-aged seed bank communities, compared to old and young seed bank communities. Nonetheless, we can conclude that no clear convergence in species composition occurs with increasing time since restoration. Other studies have also observed the absence of convergence, and even increasing divergence of the seed bank species composition with progressing time in temperate forests (Van Calster *et al.* 2008; Royo & Risteau 2012) and calcareous grasslands (Willems & Bik 2008).

At the trait level, however, a significant decrease in differentiation between middle-aged and old seed bank communities occurred. The same trend, although not significant, is present for young seed bank communities, showing higher differentiation

than old seed bank communities. Since young grassland patches were only available in 4 of the 7 sampled grasslands, only a small number of pairwise *grassland x grassland* differentiation values was obtained for young grassland patches, possibly explaining why no significant effects were observed. For this reason we believe that, at least to a certain extent, predictable changes in trait composition occur with progressing time since restoration, resulting in trait convergence in time. These results are in accordance with patterns observed in the above-ground vegetation of our study area, where trait convergence was observed, whereas at the species level, communities remained differentiated (see chapter 3).

5.5.3 SPATIAL EFFECTS

When looking solely at the seed bank composition of old grassland patches, significant isolation by distance is observed at the species level. For young and intermediate-aged grassland patches we observed no effects of spatial configuration. These results can be anticipated, since the soil seed bank community of old grassland patches contains mainly specialist species. Unlike the omnipresent generalist species, specialist species are often confined in their distribution across the landscape and often exhibit limited dispersal capacity (Thompson *et al.* 1999). For this reason we can expect spatial configuration to have large effects on the distribution of these species in the above-ground vegetation (see chapter 2), and consequently on their soil seed bank, turning the seed bank communities spatially contingent.

At the trait level, we observed no significant effects of the spatial configuration, confirming that functional assembly and changes in the functional composition of the seed bank is not governed by its location in the landscape. Previous research, however, has observed effects of spatial isolation on seed bank assembly, with strong isolation resulting in a slowdown of assembly rate in both above and below-ground assembly on restored calcareous grasslands (Fagan *et al.* 2010), a pattern also observed in the above-ground assembly of our study area (see chapter 2).

5.5.4 COMMUNITY DISASSEMBLY VS. ECOLOGICAL PALIMPSEST

Our results support the 'community disassembly' hypothesis (Zavaleta *et al.* 2009) rather than the 'ecological palimpsest' hypothesis (Davies & Waite 1998). To our knowledge this is the first study providing proof for this hypothesis during semi-natural grassland assembly. This implicates that the seed bank species composition of old patches forms a nested subset of the seed bank species composition of young patches. This suggests that after initial restoration, the open soil acts as a seed recipient, quickly building up a large seed bank within the first years after restoration, followed by a stage of gradual net loss of species while time progresses (Fagan *et al.* 2010). However, unlike observed in forest studies, our results suggest that this species loss was not governed by seed traits promoting seed survival (Van Calster *et al.* 2008; Plue *et al.* 2010; Royo & Risteau 2012), but rather seems to be driven by changes in the above-ground vegetation (Falinska 1999; Bekker *et al.* 2000; Rosef 2008). This idea is further supported by the relatively high similarity among the above ground vegetation and the soil seed bank in grasslands (Sørenson's similarity index = 54 ± 2.7% ; Hopfensperger 2007). This leads to the replenishment of seeds of specialist grassland species, but a depletion of the diverse therophyte community that dominates the seed bank of young calcareous grasslands (cf. Bekker *et al.* 2000; Bisteau & Mahy 2005; Rosef 2008; Fagan *et al.* 2010). This results in the contra-intuitive decrease in mean seed bank longevity with increasing time since restoration.

Nevertheless, this disassembly process does not result in a decrease in species differentiation among seed bank plots through time, suggesting that the 'nested subset' of seed bank species present at old grassland patches is not necessarily the same at each site (Plue *et al.* 2010). This is further confirmed by the occurrence of a significant effect of spatial configuration on the seed bank differentiation among ancient grasslands, indicating differential species compositions between geographically distant seed bank plots. A similar pattern of nested species composition of old sites, but higher differentiation among old sites has been observed for temperate hardwood forests in the USA (Royo & Risteau 2012). The authors of this study hypothesize that this increased differentiation is caused by the occurrence of several stochastic factors, partly undermining deterministic assembly patterns. More specifically these stochastic factors are identified as being: 1. inter-site variation in establishment and extinctions of species from seeds among locations; 2. long distance colonization events; and

3. *in situ* colonization of seeds from local reproduction events (Royo & Risteau 2012). The same patterns likely apply to our study system. At the functional trait level, however, this 'end state' composition seems to be more deterministic, leading to a decrease in differentiation among seed bank plots in trait composition and no effects of spatial configuration on the seed bank trait differentiation.

CHAPTER 6.

GENERAL DISCUSSION AND CONCLUSIONS

6.1 OUTLINE OF MAIN RESULTS

From the initial deterministic climax model of succession (Clements 1916) that was quickly criticized by Gleason (1927), to more recent ideas of more stochastic and contingent models of community assembly (e.g. Drake 1991; Samuels & Drake 1997; Young *et al.* 2001; Chase 2003; Vellend 2010); community assembly theory has come a long way. In this study, we evaluated the predictions of the contingent theory of community assembly in an ecological restoration context. More specifically, we examined the effects of restoration age on different levels of diversity organization, by looking at the species and functional trait level of both the above ground plant and soil seed bank communities. Furthermore, we attempted to bridge the gap between community and population ecology by comparing colonization patterns of one model species (*Origanum vulgare*) following restoration, with above ground community assembly patterns (Vellend & Geber 2005). Since our study system consisted of a number of isolated calcareous grassland fragments, we were able to incorporate the effects of spatial configuration of the fragments on the observed assembly patterns at the different organization levels of biodiversity. This allowed us to focus on two largely ignored topics regarding community assembly during ecological restoration: (i) the evaluation of assembly at both the functional trait and the gene level; and (ii) the incorporation of the effects of habitat configuration on community assembly (cf. Montalvo *et al*. 1997; Lindborg *et al*. 2008; Brudvig 2011).

A schematic overview of the main results of this PhD study is presented in Table 6.1. Strongest assembly driven changes in diversity were observed at the trait level of both the above ground plant community and the seed bank (chapters 2 & 5), with less clear patterns at the species and gene levels (chapters 2, 4 & 5). Remarkably, changes in differentiation among grassland patches with progressing assembly were different between the species and trait level (chapters 3 & 5). At the species level, for both the above ground and the seed bank community, differentiation remained relatively constant. At the trait level, however, strong convergence in trait composition was observed for both the above ground and the seed bank community. At the gene level, mean pairwise genetic differentiation among populations of *O. vulgare* was significantly larger for old populations on ancient grasslands, than for restored populations (chapter 4). The spatial configuration of the grassland fragments was observed to strongly affect (i) the assembly rate of the above ground community, (ii) gene flow patterns in *O. vulgare*; and (iii) the seed bank composition of the ancient grassland patches (chapters 2, 4 & 5).

Table 6.1. Overview of the main results of the temporal and spatial effects classified according to diversity organization level, with the occurrence of priority effects expected to occur at the species and gene levels, but not at the functional trait level. Ch. = chapter.

		Community	
		Species	Traits
Temporal (age)	Diversity	No change in sp. richness, significant changes in composition (Ch.2)	Strong directional patterns (Ch.2)
	Differentiation	No change through time, evidence for divergence (Ch.3)	Convergence through time (Ch.3)
Spatial	Spatial configuration/ isolation	Slow down of assembly (Ch.2)	Slow down of assembly, isolation acts as a trait filter (Ch.2)

6.2 DRIVERS OF COMMUNITY ASSEMBLY REVISITED

6.2.1 REGIONAL SPECIES POOL

We were unable to include the effects of variability in the regional species pool on assembly patterns in our analyses, since this PhD study was limited to one study area. The regional species pool is, however, expected to solely affect assembly at the species level, not at the trait level (Petermann *et al*. 2010). Indeed, functional traits are believed to be directly governed by abiotic and biotic factors, independent of the regional species pool (McGill *et al.* 2006; Matthews *et al*. 2009). We can thus assume that the trait assembly patterns observed in this study remain relevant and applicable to unrelated grassland systems. These assumptions should, however, be treated with caution. It is indeed uncertain to what extent these generalizations will remain applicable (Westoby 1999). For example, we can quite confidently assume that the trait patterns observed in this study are applicable to other calcareous grasslands outside of our study area, however, whether they will be applicable to other grassland types remains a question for further research.

Population	Soil seed bank	
Genes	Species	Traits
No change in genetic diversity, higher F_{IS} in old populations (Ch.4)	Decrease in sp. richness, significant changes in composition (Ch.5)	Strong directional patterns (Ch.5)
Larger genetic differentiation among old populations, no founder effects (Ch.4)	No change through time (Ch.5)	Convergence through time (Ch.5)
Gene flow spatially structured, significant isolation by distance for old populations (Ch.4)	Significant isolation by distance for the seed bank of ancient grasslands (Ch.5)	No effects (Ch.5)

6.2.2 SITE-LEVEL CONDITIONS

It is widely established that site-level conditions have strong effects on community assembly and vegetation structure (Cottenie 2005). Indeed, our study system (grasslands of the *Brometalia erecti* order) is characterized by a specific set of species, which are in turn confined in their distribution by a specific set of environmental and climatic conditions (high soil calcium concentration, low soil nutrient content and low water retention capacity) (Calaciura & Spinelli 2008). For this reason, we did not directly include the effects of site-level conditions on assembly as a scope in this study, although small-scale variations in soil depth and plot inclination were observed to affect species assembly patterns (chapter 2). At the gene level, site-level conditions are predicted to affect population formation by causing fitness differences among genotypes (Alexander *et al*. 2012). Since we focused on putatively neutral genetic markers in this study, site-level conditions have not confounded our results (chapter 4).

6.2.3 LANDSCAPE CONTEXT

We observed strong effects of landscape configuration on assembly patterns. Most strikingly, our results showed a slowdown of assembly at both the species and the trait level on strongly isolated grassland patches (chapter 2). This suggests that colonization of grassland species will be delayed under strong isolation, a hypothesis partly supported by the genetic analyses on *Origanum vulgare* (chapter 4). Gene flow during colonization of *O. vulgare* was indeed spatially structured, with colonization occurring mainly from nearby source populations. This gene flow was nevertheless considerable, as it led to a successful colonization of *O. vulgare* on many restored grasslands, eliminating pronounced spatial effects on genetic diversity (isolation by distance). However, spatial isolation was observed to act as a dispersal trait filter on plant community assembly (chapter 2). This suggests that species exhibiting the optimal set of dispersal traits will not be strongly impeded by spatial isolation during colonization (Lindborg *et al*. 2011). *O. vulgare* was observed to show this optimal trait set, consisting of relatively small and light seeds with high attachment potential, and large plant size (Table 6.2) (chapters 2

& 4). We can expect that species with contrasting dispersal trait sets will be impacted more severely by spatial isolation, resulting in strong effects on genetic diversity and differentiation during colonization.

The impact of spatial isolation on species colonization is also dependent upon the species' specific hostility of the landscape matrix (Manel *et al*. 2003). Most generalist species, for example, are not confined to calcareous grassland remnants, but also occur in parts of the landscape matrix, resulting in a much lower effective isolation of their populations (Thompson *et al*. 1999). Generalist species were indeed observed to be the first to appear at restoration sites, with even positive effects of increased calcareous grassland isolation (chapter 2). This can, in turn, explain the absence of an isolation by distance pattern for the seed bank communities of restored grasslands (chapter 5).

Table 6.2. Overview of traits filtered by isolation (chapter 2). Trait values given for *Origanum vulgare* and mean value for all non-woody species observed in this study (sp. mean).

	O. vulgare	*sp. mean*
plant height (m)	0.55	0.48
seed length (mm)	0.85	2.24
seed mass (mg)	0.09	4.66
A.P. sheep (%)	63.95	56.39
A.P. cattle (%)	15.63	9.94

The seed bank species composition of ancient grasslands was shown to be spatially structured, indicating that the effects of grassland isolation can persist in end state grassland communities (chapter 5). This can be explained by the fact that the seed bank composition of ancient grasslands consisted mainly of isolation-prone specialist species (Thompson *et al*. 1999). This persistence of isolation effects on the end state community was furthermore confirmed by the significant isolation by distance pattern of old *O. vulgare* populations (chapter 4) and the loss of specialist species from isolated, ancient grassland patches (Adriaens *et al*. 2006). At the trait level, however, seed bank composition of both old and restored grassland patches was unaffected by spatial isolation (chapter 5). These results, combined with the results of the above ground community (chapter 2), suggest that isolation merely slows down predictable trait assembly, rather than change its trajectory. However, note that these conclusions hold for traits related to niche determination, rather than traits relating to dispersal, with the latter clearly being filtered by isolation (chapter 2).

6.2.4 HISTORICAL FACTORS

Although demonstrating the occurrence of priority effects was not a direct objective of this study, we found indications that suggest contingencies within the studied communities. At the species level, communities of different grassland fragments were observed to remain differentiated, or even to diverge with progressing assembly (chapter 3). This suggests that initial differences in species composition, caused by small scale variation in site-level conditions, landscape effects and/or historical factors (Brudvig 2011), persist throughout the assembly process through the action of priority effects and dispersal limitation (Petermann *et al*. 2010). A similar pattern was observed in the soil seed bank, with species differentiation remaining constant with progressing assembly (chapter 5).

Historical effects can manifest themself in the form of founder effects at the genetic level. However, we did not observe genetic founder effects in young populations of *O. vulgare* (chapter 4), most likely due to high levels of among population gene flow, accompanied by considerable colonization events from multiple source populations. Strong founder effects are indeed more likely to occur in species with lower colonization capacities, with population foundation occurring through only a limited number of colonization events (Slatkin 1977; Whitlock & McCauley 1990).

In accordance with theoretical predictions, assembly of both the above ground vegetation and the soil seed bank was unaffected by contingencies at the trait level (Kahmen & Poschlod 2004; Fukami *et al*. 2005). Progressing assembly was characterized by a reduction in trait dissimilarity among grassland patches for both the above ground and seed bank community (chapters 3 & 5). As discussed previously, this trait convergence suggests that niche space filling is defined by species functional traits rather than species identity. Indeed, assuming the presence of similar niches within environmentally similar sites and the occurrence of multiple species for each given niche within the regional species pool explains the co-occurrence of trait convergence and of species divergence (Fukami *et al*. 2005; Cleland *et al*. 2011).

It has been suggested that the presence of a soil seed bank can induce priority effects through manipulation of the species arrival order at an assembly site (Willems & Bik 1998). We were unable to evaluate this hypothesis for two reasons. First of all,

we do not know what grassland species persist in the seed bank of forested grassland sites. Thus, when looking at the seed bank composition of restoration sites, we are unable to differentiate between remnant seeds that originated prior to afforestation, and seeds that colonized after restoration took place. Second, to truly assess the effects of the seed bank on vegetation assembly, an experimental approach in which the soil seed bank is removed from several grasslands would be more suited. This would allow the comparison of plant community assembly among grasslands with and without a soil seed bank. This approach, however, implies considerable logistic investments, making it unfeasible to be performed within the scope of this PhD.

Previous research in these and in related grassland types, however, established that the soil seed bank under *Pinus* forests is largely devoid of species relevant for grasslands assembly (Bisteau & Mahy 2005; Bossuyt *et al*. 2006; Jacquemyn *et al*. 2011). For this reason, we believe that the priority effects induced by the remnant seed bank are very limited in our study system. Nevertheless, more research is needed to truly understand the potential impact of seed bank induces priority effects on above ground assembly.

6.3 SIMILARITIES AND DIFFERENCES ACROSS ORGANIZATIONAL LEVELS OF DIVERSITY

We observed strong similarities in assembly patterns of the above ground community and the soil seed bank, at both the species and the functional trait level (chapters 2,3 & 5) (Klimkowska *et al*. 2010). Indeed, progressing assembly of both the above ground and seed bank communities involved a sequential replacement of generalist by specialist species, translated at the trait level by a replacement of early flowering, large seeded, hemerochore species of nutrient rich soils by insect pollinated, later flowering specialist species of nutrient poor soils, rosette species and epizoochore species. This suggests that the changes in the above ground community have considerable effects on the species composition of the seed bank, which is in accordance with the typical observation of relatively high similarity among the above ground vegetation and the soil seed bank in grasslands (Hopfensperger 2007). Nevertheless, we also observed seed bank assembly patterns that were independent of the above ground community

assembly, such as age-structured nestedness, and a decrease in total species richness through time (chapter 5). This suggests a fast initial buildup of a diverse seed community, followed by the gradual loss of species. We can interpret these patterns as a time lag between changes in the above ground community and the seed bank, and a gradual filtering of species in the seed bank, opposed to the sequential replacement observed in the above ground vegetation (Royo & Risteau 2012). Minor differences in responding traits occurred between the above ground community and the seed bank at the trait level, suggesting that forces other than above ground assembly are driving functional seed bank patterns. These results suggest that seed bank assembly and composition is more than just a mere reflection of the above ground vegetation and that it indeed should be studied in its own right, to fully understand community assembly in all its aspects.

Our results do not fully support the theoretical prediction that community assembly and population colonization show parallel patterns in diversity and differentiation through time (Vellend & Geber 2005; Alexander *et al*. 2012). Indeed, young *O. vulgare* populations seemingly instantly built up high genetic diversity, eliminating any founder effects or strong genetic differentiation (chapter 4). The literature, however, shows that, unlike community priority effects, the occurrence of strong initial founder effects are likely quite limited in nature and strongly affected by the degree of gene flow (Slatkin 1977; Whitlock & McCauley 1990). Most successfully colonizing species have indeed been observed to exhibit large genetic diversity within their founder populations (e.g. Erickson *et al*. 2004; Pluess & Stöcklin 2004; Raffl *et al*. 2006; Yang *et al*. 2008). Nevertheless, patterns of genetic diversity have been observed to be affected by species functional traits (Duminil *et al.* 2009). Thus, we can assume that the occurrence of population founder effects is strongly species dependent, determined by its dispersal and establishment trait set, making it very hard to generalize colonization patterns using only one species (Vellend & Geber 2005).

Even if initial founder effects occur in neutral marker allele frequencies, they are unlikely to persist in time (Holderegger *et al*. 2010). Since alleles of neutral markers are interpreted as phenotypically equivalent, the presence of one allele in the population does not prevent colonization of other alleles, thus explaining the importance of gene flow for founder effect occurrence and severity (Holderegger *et al*. 2010). This point embodies the fundamental difference between genetic founder effects and community priority effects, since species are not considered equivalent during assembly, enabling early arriving species to inhibit or to facilitate the colonization of later arriving species

(Chase 2003; Trowbridge 2007). Adaptive genetic diversity, on the other hand, might show very different patterns, with possibly persistent priority effects shaping population dynamics (Vellend & Geber 2005). However, the evaluation of adaptive genetic variation was beyond the scope of this study.

The framework of Vellend (2010), which discusses four major processes shaping both communities and populations (chapter 1), can be applied to this study. Although not examined here, the processes of *mutation and speciation* undoubtedly affect assembly and colonization by defining the regional species/gene pool. Our results suggest that *selection*, hypothesized to drive *all* deterministic patterns, clearly acts at the functional trait level rather than the species level. At the gene level, in turn, the effects of *selection* can only be observed when looking at quantitative genetic variation, rather than at neutral variation. *Drift* was clearly observed at the species level, with large differentiation among restoration sites. In addition, at the genetic level, *drift* likely led to the increased differentiation among old populations of *O. vulgare*. *Immigration (dispersal/gene flow)*, finally, was observed to affect both community assembly and species colonization, as was previously discussed in this chapter.

It is also worth noting that the decline in generalist species diversity is expected to be reflected in a loss of genetic diversity within these species. Accompanied by an increase in genetic drift, causing genetic differentiation, this decline in genetic diversity can be expected to result in the loss of these populations through inbreeding effects (Keller & Waller 2002; Honnay 2013). Differences in assembly patterns among the species and trait level have already been described in detail previously and will not be further discussed here.

6.4 IMPLICATIONS FOR RESTORATION

Although this study mainly focuses on the theoretical aspects of community assembly, many of our results can be used to generate restoration guidelines applicable to other, unrelated calcareous grassland systems, and possibly even to other grassland types (Figure 6.1).

1. Apart from taking into account well-established factors such as local abiotic conditions and the regional species pool when designing restoration schemes, it is important to take historical factors and the landscape configuration into account (cf. Brudvig 2011). More specifically, our results suggest that restoration success will benefit from nature conservation actions that reduce dispersal limitation. This can partly be accomplished through increased movement of cattle or sheep between restoration patches (Adriaens *et al.* 2007; Hedberg & Kotowski 2010). Although seed exchange by migrating grazers likely facilitated seed dispersal in our study area, our results suggest that this is insufficient to compensate for the isolation effects on the species composition. Therefore, restoration would benefit from physically interconnecting grassland fragments or from introducing seeds or seedlings, especially of species with low dispersability (see Brudvig 2011). Taking into account dispersal trait suits of target species will also help to predict the likelyhood of spontaneous colonization of these species, and to help decision making regarding assisted colonisation.

Acknowledging the effects of contingencies, acting through historical land use, habitat configuration, and priority effects will help to anticipate unpredictable changes in species composition and species divergence among restoration sites (Purschke *et al*. 2012). These seemingly random patterns will become more predictable by also adopting a trait-based restoration approach next to the traditional focus on the species level, as demonstrated in our study. By looking at the functional trait composition of the target community, an end state trait suit can be described to help evaluate what species (combinations) can be expected to occur at the restoration site (Pywell *et al*. 2003; Douma *et al*. 2012). Although we did not quantify the trait set of the target (ancient grassland) community in this study, we did observe an increase in species with traits associated with nutrient

acquisition, drought tolerance and high light availability, suggesting what species will likely occur at the restoration sites in time (chapter 2). The other way around, these trait suits can be used to identify undesirable species at the restoration site, potentially competing with target species for a certain trait-defined niche space, which subsequently could explain the absence of these target species. This information can be used to perform effective targeted manipulations of the species composition at the restoration site to facilitate the establishment of certain target species. This insight will also lead to a better understanding of the potentially great impact of introducing species to newly restored sites through seed or seedling addition, since they may exhibit strong priority effects on community assembly, possibly leading to the absence of several target species (Funk *et al.* 2008).

2. One colonization event at the restoration site does not necessarily imply the formation of a sustainable population. Especially for rare target species exhibiting poor dispersal trait suits, such as *Anemone pulsatilla, Cirsium acaule* or *Gentianella germanica* in our study area, it is useful to evaluate the number of potential source populations and their genetic composition. Using this information, restoration should try to maximise gene flow from these putative source populations toward the restoration site, by optimising connectivity (Segelbacher *et al.* 2010). Hence, a sufficient number of colonisation events from a considerable number of source populations can be effectuated in an attempt to overcome deleterious founder effects (Slatkin 1977; Whitlock & McCauley 1990). If the genetic viability of these source populations is poor, it might be advisable to actively introduce seeds from other, regionally present, genetically diverse populations in an attempt to establish new, viable populations and reconnect the declining remnant populations in a sustainable metapopulation (Alexander *et al*. 2012; Reynolds *et al*. 2013). The possible presence of the target species in the soil seed bank should also be evaluated. Indeed, in this study we observed the presence of several calcareous grassland target species in the seed bank, such as *Genista sagittali, Globularia punctata* and *Scabiosa columbaria*, among others (chapter 5). The seed bank of these species could contain genetic diversity previously lost from the above-ground vegetation, which could lead to more diverse, less differentiated founder populations through germination, as was observed for *O. vulgare* in this study (chapter 4) (Honnay *et al*. 2008; Mandák *et al*. 2012).

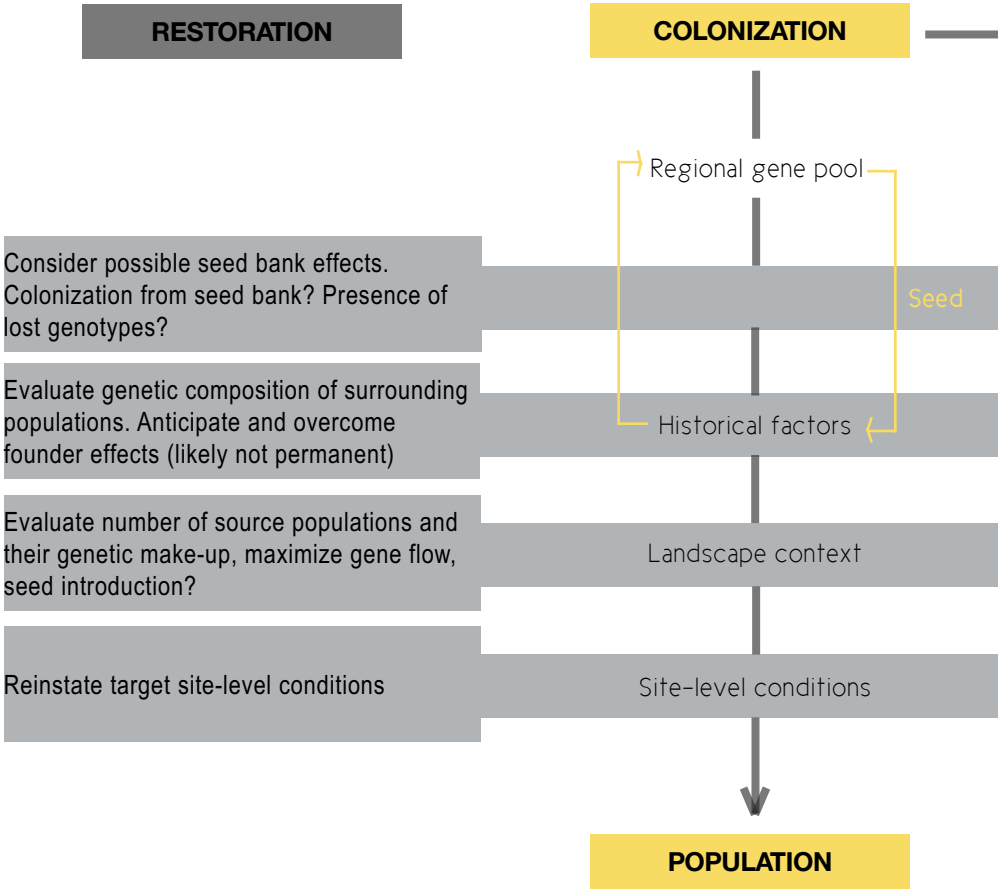
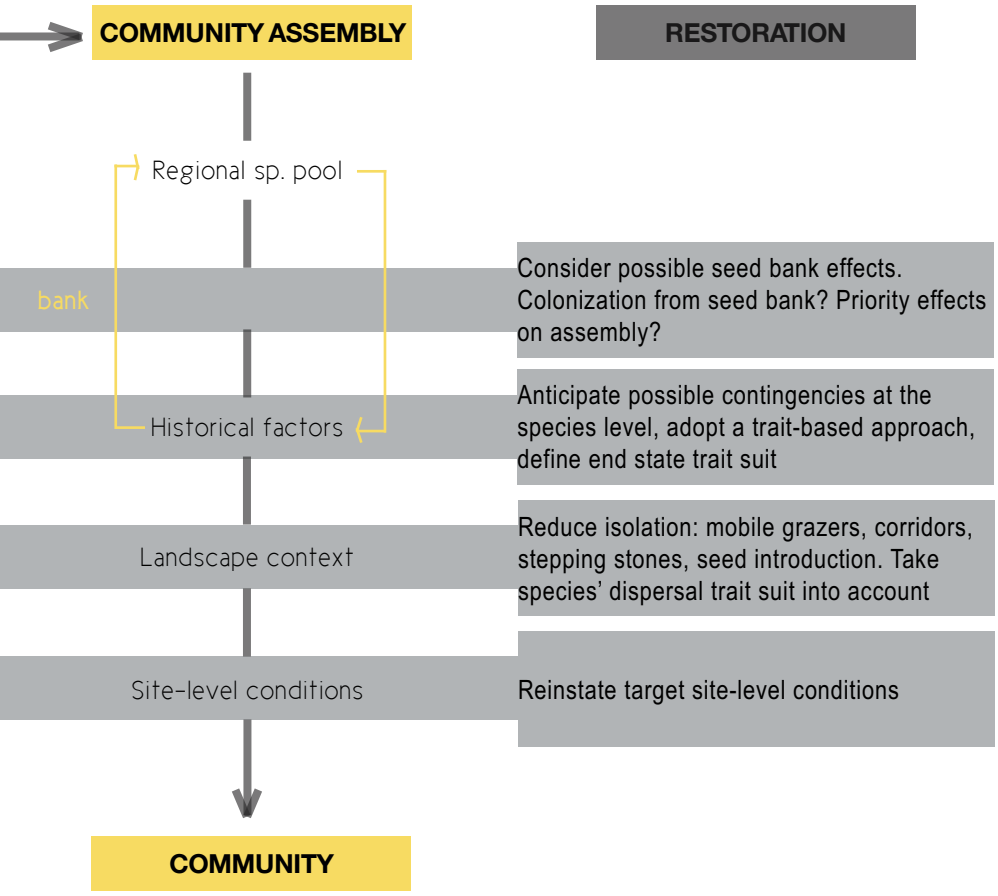


Figure 6.1. Schematic overview of the implications for restoration based on our results. Figure follows the structure of figure 1.8 in the introduction.

3. The soil seed bank should be taken into account during habitat restoration. Evaluating the historical vegetation history and species composition of the site prior to restoration, using historical maps or vegetation data can help anticipate which species are likely present in the seed bank. The occurrence of certain dominant generalist species in the seed bank can strongly affect initial assembly through priority effects (Grman & Suding 2010). The other way around, presence of remnant seeds of target species in the seed bank can result in fast, successful colonization and establishment of viable populations of these species (Bossuyt &



Honnay 2008a). Although our results do not result in clear conclusions regarding the restoration potential of the seed bank, they do indicate that seed bank formation is a far more complex process than just a reflection of what is going on in the above ground plant community. Thus, we can expect that the seed bank can have effects on the above ground community assembly, through priority effects, buffering of population dynamics and acting as a genetic reservoir (Kalamees & Zobel 2002; Honnay *et al*. 2008; Mandák *et al*. 2012).

6.5 SHORTCOMINGS AND RESEARCH PERSPECTIVES

1. Since this study was performed in a single study system, it is hard to assess the generality of the observed patterns. Replication of these analyses over different study systems will overcome this problem and could furthermore lead to a quantification of the relative importance of the regional species pool, site-level conditions, the landscape context and historical factors on community assembly. Particularly the effects of regional species pool and site-level conditions could greatly contribute to the insights gained from this study. Comparison of trait assembly between contrasting vegetation types could furthermore indicate how similar trait assembly patterns are across vegetation types.

2. Limitations of the use of chronosequences in community ecology are widely acknowledged (Foster & Tilman 2000; Johnson & Miyanishi 2008; Walker *et al*. 2010). Indeed, the level of detail at which successional patterns can be predicted will be limited because of the regional averaging that occurs (Foster & Tilman 2000). Secondly, it is impossible to ensure that the observed successional changes are solely caused by time and not affected by other co-varying local factors (Foster & Tilman 2000; Walker *et al*. 2010). For this reason, it would be beneficial to replicate our experiments using permanent plot observations. This would eliminate possible effects of microsite abiotic variation and land use history from the observed assembly patterns, likely increasing the predictive power of the statistical models.

3. During this PhD, we were able to only investigate colonization patterns of a single grassland species. However, to be able to generate general predictions regarding the processes shaping the occurrence and severity of genetic founder effects during colonization, this analysis should be complemented with the genetic analysis of other colonizing grassland species with contrasting functional trait suits, such as *Hippocrepis comosa* or *Anthyllis vulneraria*, which are known to lack a persistent seed bank and have lower dispersal capacities than *Origanum vulgare*, but have nonetheless established several new populations on restored grasslands.

4. The presence of priority effects was only indirectly inferred in this study. However, we also adopted an ongoing experimental approach to directly quantify the effects of priority effects on community assembly. In this experiment, assembly was allowed from three different starting conditions, consisting of the removal of one certain functional group (graminoids, legumes and control) within a 5 x 5 m plot. This set-up was replicated over four grasslands, with four replications of each manipulation within each grassland fragments, adding up to 48 plots. After initial manipulation, these communities were allowed to follow spontaneous community assembly. Using the results of this experiment will allow direct quantification of the severity of priority effects on ongoing community assembly in our study area.

5. Phylogenetic diversity patterns have gained much attention recently. According to recent theory, many functional traits show phylogenetic conservatism (Prinzing *et al*. 2001; Hardy & Senterre 2007), implicating that phylogenetic relatedness among species within a community can partly explain the functional composition of this community (Devictor *et al*. 2010). For this reason, assembly patterns are expected to be more predictable at the phylogenetic level as opposed to the species (taxonomic) level, resulting in clear restoration guidelines (Verdú *et al*. 2012). The predictive power of trait analyses are furthermore heavily dependent on the subset of functional traits used, a problem that is believed not to occur using phylogenetic analyses (Cadotte *et al*. 2013). Several recent studies, however, suggest that a functional approach has better predictive power than a phylogenetic approach (Bennett *et al*. 2013; Carboni *et al*. 2013; Purschke *et al*. 2013). Nonetheless, evaluating assembly in a phylogenetic context, combining the functional trait and phylogenetic framework, will almost certainly result in novel insights (Webb *et al.* 2008; Cadotte *et al*. 2013). Also the analysis of changes in functional diversity throughout assembly and its effects on ecosystem functioning and stability remain interesting research opportunities (Díaz *et al.* 2007; Cadotte *et al.* 2011).

6. Also the use of database extracted functional trait states has its limitations (Kazakou *et al*. 2013). Measuring different species trait values *in situ* and allowing for intra-species trait variation would likely improve the predictive power of our statistical models and lead to better insights of how assembly is shaped by community trait composition (Laughlin *et al*. 2012; Violle *et al.* 2012; Kazakou *et al*. 2013).

7. All vegetation patterns were derived using only two 2 x 2 m plots for every restored hectare in this study (chapters 2 & 3). Using a more intensive sampling design and the collection of species presence data for each grassland patch would undoubtedly lead to clearer insights in the different processes shaping community assembly on these grasslands. Furthermore, we decided not to include the effects op grassland patch area in our analyses for two reasons. First of all, we worked with plot-based data rather than grassland patch data, which are likely less affected by patch area. Secondly, different grassland patches are connected in larger grassland fragments, making it hard to decide how grassland area should be defined to be ecologically relevant (patch area or fragment area). Nevertheless it would remain interesting to evaluate possible effect of grassland patch/fragment area on community composition and assembly, by performing plant presence surveys in every grassland patch. This would allow a more direct analysis of the effects of patch area on community assembly.

8. The evaluation of the occurrence of local trait clustering or trait overdispersion, caused by the relative importance of environmental filtering opposed to competition, would also lead to valuable insights in the patterns driving community assembly. The occurrence of trait clustering would furthermore support our hypothesis of limited niches occurring at the trait level, driving the deterministic trait convergence among grassland patches through time (chapter 3) and thus strengthen our conclusions.

APPENDICES

Appendix 2.1. Spearman rank correlations of abiotic and spatial variables to restoration age and closest edge distance. Test statistic and Spearman *R* given. Spatial autocorrelation analyses for age and clostest edge. Test statistic and Moran's I given. Variables were calculated for each individual grassland patch (n = 46). Significance: *0.05 ≥ *P*-value > 0.01 **0.01 ≥ *P*-value > 0.001 ***0.001 ≥ *P*-value.

	age		closest edge	
	t	R	t	R
% bare rock	-1.6	-0.23	-0.8	-0.083
% open soil	-2.2*	-0.31	-0.6	-0.083
soil depth	0.1	0.021	2.4*	0.33
plot inclination	-1.4	-0.20	-2.4*	-0.34
patch elevation	0.5	0.067	0.5	0.082
isolation: closest edge	-0.6	-0.093	-	-
isolation: closest centroid	-0.9	0.13	9.0***	0.80
isolation: buffer	1.7	0.25	-2.1*	-0.36
isolation: Hanski	0.5	0.072	-2.0*	-0.32
	Z	Moran's I	Z	Moran's I
spatial autocorrelation	0.2	-0.007	2.0*	0.11

trait	description	scale	% of data available	data source	ch. 2&3	ch. 5
life form	1. phanerophyte; 2. chamaephyte; 3. hemicryptophyte; 4. geophyte; 5.therophyte	nominal	97.2	Klotz *et al.* 2002		x
plant height	mean individual height (m)	ratio	100	Lambinon *et al.* 1998	x	x
life span	1. annual; 2. biennial; 3. once flowering perennial; 4. multiple flowering perennial	nominal	100	Klotz *et al.* 2002	x	x
rosette type	1. rosette; 2. half-rosette; 3. no rosette	nominal	100	Klotz *et al.* 2002	x	x
L	Ellenberg light value: ranging from 1 (plants of deep shade) to 9 (plants of full sun)	ordinal	97.2	Kleyer *et al.* 2008	x	x
N	Ellenberg nutrient value: ranging from 1 (plants of nutrient poor soils) to 9 (plants of nutrient rich soils)	ordinal	85	Kleyer *et al.* 2008	x	x
flowering start	Month in which flowering begins	ordinal	99.6	Klotz *et al.* 2002	x	x
pollen vector	1. insects; 2.selfing; 3. wind; 4. water	nominal	96.4	Klotz *et al.* 2002	x	x
reproductive type	1. mainly seeds/spores; 2. seeds/spores & vegetative; 3. mainly vegetative	nominal	99.6	Klotz *et al.* 2002	x	x
diaspore type	1. fruit; 2.seed; 3. spore; 4. vegetative	nominal	100	Klotz *et al.* 2002	x	
seed length	length of a single seed (mm)	ratio	90.3	Kleyer *et al.* 2008	x	x
seed shape	seed length/width ratio	ratio	87	Kleyer *et al.* 2008	x	x
seed longevity	proportion of short and long term persistent seed records on total number of records per species	ratio	83.8	Thompson *et al.* 1997, Bekker *et al.* 1998	x	x
fertility system	1. autogamous; 2. mixed mating system; 3. allogamous	nominal	86.2	Klotz *et al.* 2002	x	x
seed mass	mass of a single seed (mg)	ratio	93.1	Kleyer *et al.* 2008	x	x
seed number	logaritmic transformed number of seeds per plant	ratio	89.9	Kleyer *et al.* 2008	x	x
mycorhizal frequency	0. never; 1. rarely; 2. occasionally; 3. normally	ordinal	77.3	Fitter & Peat 1994	x	x

mycorhizal type	1. arbuscular; 2. ecto; 3. orchid (chapter 2 & 3); 3. ericoid (chapter 5)	nominal	74.1	Fitter & Peat 1994	x	x
autochory	0. no autochory; 1. Self dispersal	binary	94.7	Kleyer *et al.* 2008	x	x
hemerochory	0. no hemerochory; 1. Seed dispersal by human action	binary	94.7	Kleyer *et al.* 2008	x	x
anemochory	0. no anemochory; 1. Seed dispersal by wind	binary	94.7	Kleyer *et al.* 2008	x	x
endozoochory	0. no endozoochory; 1. Seed dispersal after digestion	binary	94.7	Kleyer *et al.* 2008	x	x
epizoochory	0. no epizoochory; 1. Adhesive dispersal by animals	binary	94.7	Kleyer *et al.* 2008	x	x
dysochory	0. no dysochory; 1. Seed dispersal by scatter-hoarding animals	binary	94.7	Kleyer *et al.* 2008	x	x
nitrogen fixation	1. nitrogen fixators; 2. non fixators	binary	100	Poschlod *et al.* 2003	x	x
Age of first flowering	1. < 1 year; 2. between 1 and 5 years; 3. > 5 years	ordinal	76.1	Kleyer *et al.* 2008	x	x
leaf size	mean leaf size (mm2)	ratio	87.9	Kleyer *et al.* 2008	x	x
A.P. sheep	Seed attachment potential to sheep wool (%)	ratio	87	Römermann *et al.* 2005, Lappers *et al.* 2006	x	
A.P. cattle	Seed attachment potential to cattle hair (%)	ratio	87	Römermann *et al.* 2005, Lappers *et al.* 2006	x	

Appendix 2.2. Overview of the selected traits, used for the trait analysis. Description, scale, percentage of data availability and main data sources for every trait given. Last two coloms indicate what traits were respecitively used for the analyses in chapter 2, 3 and 5.

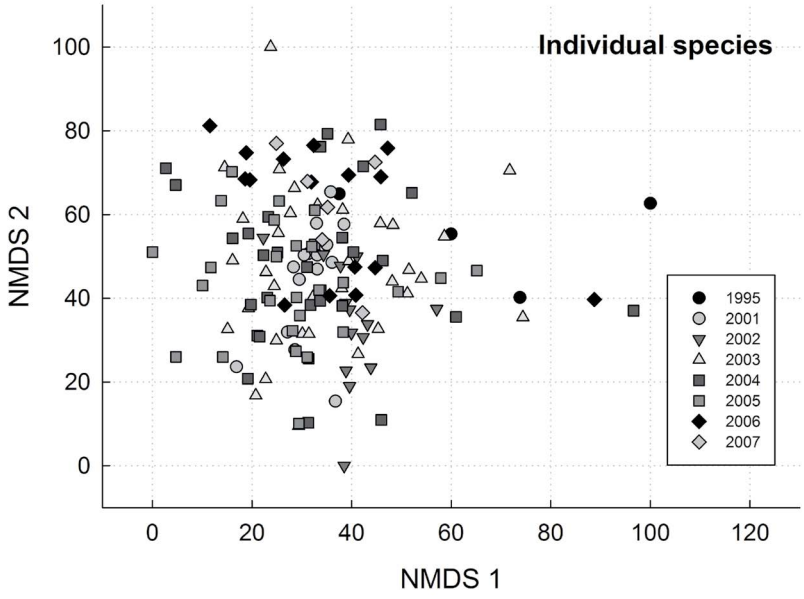
Appendix 2.3. Overview of the species list. Occurrence frequency (freq.) (% of occupied plots) of the species given. Emergent group identity given for every species. Group numbers correspond to the emergent groups described in Table 2.2; group 1: Megaphanerophytes, group 2: Forest/ shrub species, group 3: Orchids, group 4: Small grassland herbs, group 5: Large herbs & grasses, group 6: Sedges & shallow soil specialists, group 7: Annuals. Every species was defined as generalist (g) or specialist (s) species.

species	freq.	group	spec/ gen	species	freq.	Group	spec/ gen
Acer campestre	11.56	1	g	*Koeleria macrantha*	1.36	5	s
Acer platanoides	0.68	1	g	*Lactuca serriola*	4.76	5	g
Acer pseudoplatanus	4.08	1	g	*Lamium galeobdolon*	1.36	2	g
Achillea millefolium	5.44	4	g	*Lapsana communis*	8.16	7	g
Agrimonia eupatoria	9.52	2	s	*Lathyrus pratensis*	0.68	2	g
Agrostis capillaris	2.72	5	g	*Lathyrus sylvestris*	2.04	4	s
Agrostis stolonifera	34.69	5	g	*Leontodon autumnalis*	17.69	5	g
Allium oleraceum	2.72	4	s	*Leontodon hispidus*	41.5	5	s
Allium sphaerocephalon	2.72	4	s	*Leucanthemum vulgare*	17.69	4	g
Alopecurus myosuroides	0.68	7	g	*Ligustrum vulgare*	10.88	2	g
Anagallis arvensis	0.68	7	g	*Linum catharticum*	40.82	7	s
Anemone nemorosa	1.36	2	g	*Lithospermum officinale*	1.36	6	s
Anthericum liliago	0.68	4	s	*Lonicera periclymenum*	2.04	2	g
Anthyllis vulneraria	14.29	4	s	*Lotus corniculatus*	70.07	4	g
Aquilegia vulgaris	2.04	2	S	*Luzula campestris*	0.68	6	g
Arabis hirsuta	7.48	6	s	*Malus sylvestris*	0.68	2	g
Arenaria serpyllifolia	9.52	7	s	*Malva moschata*	1.36	6	s
Arrhenatherum elatius	4.76	4	g	*Medicago lupulina*	39.46	4	g
Arum maculatum	4.76	2	g	*Melica ciliata*	7.48	4	s
Asplenium ruta-muraria	2.72	4	s	*Melica nutans*	5.44	2	s
Asplenium trichomanes	0.68	6	s	*Melica uniflora*	0.68	2	g
Atropa bella-donna	2.04	2	s	*Melilotus altissimus*	0.68	6	g
Betula pendula	5.44	1	g	*Mercurialis perennis*	6.12	2	s
Betula pubescens	4.08	1	g	*Moehringia trinervia*	0.68	7	g
Brachypodium pinnatum	76.19	5	s	*Myosotis arvensis*	14.97	7	g
Brachypodium sylvaticum	2.04	5	g	*Neottia ovata*	0.68	3	s
Briza media	5.44	4	s	*Ophrys apifera*	1.36	3	s
Bromus erectus	1.36	5	s	*Ophrys insectifera*	1.36	3	s
Bromus hordeaceus	1.36	5	g	*Orchis mascula*	9.52	3	s
Bromus sterilis	6.8	5	g	*Orchis purpurea*	0.68	3	s

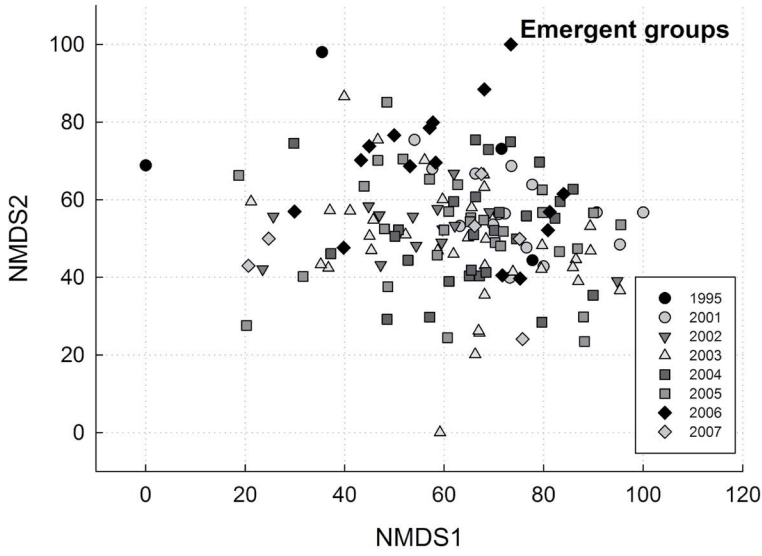
Bupleurum falcatum	2.04	4	s	*Origanum vulgare*	42.18	4	s
Buxus sempervirens	1.36	2	s	*Orobanche teucrii*	1.36	3	s
Campanula rotundifolia	21.09	4	g	*Papaver rhoeas*	0.68	7	g
Cardamine hirsuta	8.16	7	g	*Pastinaca sativa*	0.68	4	s
Carduus crispus	2.04	5	g	*Picea abies*	0.68	1	g
Carduus nutans	0.68	5	s	*Picris hieracioides*	4.08	5	s
Carex caryophyllea	34.69	6	s	*Pimpinella saxifraga*	11.56	4	s
Carex digitata	2.04	6	s	*Pinus sylvestris*	4.08	1	g
Carex filiformis	1.36	6	s	*Plantago lanceolata*	36.05	4	g
Carex flacca	85.71	6	s	*Plantago major*	9.52	5	g
Carex humilis	2.72	6	s	*Plantago media*	0.68	4	s
Carex montana	4.08	6	s	*Platanthera bifolia*	0.68	3	s
Carex muricata	0.68	6	g	*Platanthera chlorantha*	9.52	3	s
Carex panicea	0.68	6	g	*Poa angustifolia*	57.82	5	s
Carex sylvatica	0.68	6	g	*Poa annua*	7.48	7	g
Carlina vulgaris	0.68	5	s	*Poa compressa*	9.52	5	s
Carpinus betulus	22.45	1	g	*Poa pratensis*	6.8	5	g
Catapodium rigidum	2.72	7	s	*Polygala comosa*	9.52	4	s
Centaurea jacea	8.16	4	g	*Polygala vulgaris*	6.8	4	s
Centaurium erythraea	2.04	5	g	*Polygonatum odoratum*	7.48	2	s
Cephalanthera damasonium	0.68	3	s	*Populus tremula*	2.72	3	g
Cerastium brachypetalum	1.36	7	s	*Potentilla neumanniana*	59.18	4	s
Cerastium fontanum	0.68	6	g	*Potentilla reptans*	2.72	4	g
Cerastium pumilum	12.93	7	s	*Potentilla sterilis*	0.68	2	g
Cerastium semidecandrum	2.72	7	s	*Primula veris*	23.81	4	s
Cirsium acaule	6.8	4	s	*Prunella laciniata*	0.68	6	s
Cirsium arvense	39.46	5	g	*Prunella vulgaris*	8.84	4	g
Cirsium palustre	0.68	5	g	*Prunus avium*	0.68	1	g
Cirsium vulgare	25.85	5	g	*Prunus spinosa*	65.99	2	g
Clematis vitalba	61.22	5	s	*Quercus robur*	23.81	1	g
Clinopodium acinos	3.4	4	s	*Ranunculus acris*	2.04	4	g
Clinopodium vulgare	3.4	4	s	*Ranunculus auricomus*	1.36	5	g
Convolvulus arvensis	3.4	4	g	*Ranunculus bulbosus*	10.2	4	s
Conyza canadensis	1.36	5	g	*Ranunculus repens*	9.52	4	g
Cornus mas	0.68	2	s	*Rhamnus cathartica*	4.76	2	s
Cornus sanguinea	40.14	2	g	*Rosa canina*	68.71	2	g
Corylus avellana	18.37	1	g	*Rosa micrantha*	1.36	4	s
Cotoneaster integerrimus	0.68	2	s	*Rosa rubiginosa*	2.72	2	s
Crataegus monogyna	85.03	2	g	*Rubus fruticosus*	69.39	4	g

Crepis biennis	17.01	5	g	*Rubus idaeus*	8.84	2	g
Crepis capillaris	8.84	5	g	*Rumex acetosa*	0.68	4	g
Cruciata laevipes	0.68	2	g	*Salix caprea*	17.69	3	g
Dactylis glomerata	36.73	4	g	*Sambucus nigra*	0.68	2	g
Daucus carota	26.53	5	g	*Sanguisorba minor*	42.18	4	s
Digitalis lutea	5.44	6	s	*Saxifraga tridactylites*	2.04	7	s
Echium vulgare	4.76	4	s	*Scabiosa columbaria*	7.48	4	s
Epilobium montanum	5.44	5	g	*Sedum acre*	0.68	6	s
Epilobium parviflorum	1.36	5	g	*Sedum album*	7.48	6	s
Erophila verna	1.36	7	s	*Sedum rupestre*	1.36	6	s
Euonymus europaeus	14.29	2	g	*Senecio sylvaticus*	0.68	5	g
Eupatorium cannabinum	3.4	5	g	*Sesleria caerulea*	7.48	4	s
Euphorbia amygdaloides	1.36	6	s	*Silene dioica*	0.68	6	g
Euphorbia cyparissias	12.93	4	s	*Silene nutans*	2.04	4	s
Fallopia dumetorum	4.08	7	g	*Silene vulgaris*	0.68	6	s
Festuca lemanii	19.05	4	s	*Solanum dulcamara*	10.2	2	g
Fragaria vesca	81.63	4	g	*Solidago virgaurea*	2.72	5	g
Fragaria viridis	4.76	6	s	*Sonchus asper*	48.3	7	g
Fraxinus excelsior	6.8	1	g	*Sonchus oleraceus*	6.12	5	g
Galeopsis tetrahit	1.36	7	g	*Sorbus torminalis*	1.36	2	s
Galium aparine	8.84	7	g	*Stachys alpina*	2.04	5	s
Galium mollugo	8.84	4	g	*Stachys officinalis*	1.36	4	g
Galium pumilum	42.18	4	s	*Stachys sylvatica*	2.04	2	g
Galium verum	6.12	4	s	*Stellaria media*	0.68	7	g
Genista sagittalis	7.48	4	s	*Taraxacum officinale*	91.16	5	g
Genista tinctoria	2.72	4	s	*Teucrium chamaedrys*	17.01	4	s
Geranium columbinum	12.93	7	s	*Teucrium montanum*	0.68	4	s
Geranium molle	1.36	7	g	*Thlaspi perfoliatum*	7.48	7	s
Geranium robertianum	5.44	7	g	*Thymus pulegioides*	8.84	4	s
Geranium sanguineum	0.68	4	s	*Tragopogon pratensis*	3.4	5	g
Geum urbanum	32.65	5	g	*Trifolium campestre*	1.36	7	s
Glechoma hederacea	1.36	2	g	*Trifolium dubium*	6.12	7	g
Globularia punctata	2.72	6	s	*Trifolium medium*	2.04	4	s
Gymnadenia conopsea	0.68	3	s	*Trifolium pratense*	4.76	4	g
Hedera helix	14.97	1	g	*Trifolium repens*	4.76	4	g
Helianthemum nummularium	32.65	4	s	*Tussilago farfara*	0.68	5	g
Helictotrichon pubescens	12.24	5	g	*Valeriana officinalis*	0.68	5	g
Helleborus foetidus	7.48	2	s	*Valerianella locusta*	3.4	7	g
Heracleum sphondylium	0.68	2	g	*Verbascum lychnitis*	6.8	5	s

Hieracium aurantiacum	0.68	5	g	*Verbascum thapsus*	0.68	5	g
Hieracium lachenalii	5.44	5	g	*Veronica arvensis*	9.52	7	g
Hieracium laevigatum	0.68	5	g	*Veronica chamaedrys*	2.72	4	g
Hieracium murorum	1.36	5	g	*Veronica officinalis*	2.04	4	g
Hieracium pilosella	13.61	5	g	*Veronica prostrata*	2.72	4	s
Himantoglossum hircinum	0.68	3	s	*Veronica serpyllifolia*	2.04	4	g
Hippocrepis comosa	34.69	4	s	*Viburnum lantana*	5.44	2	s
Holcus lanatus	22.45	5	g	*Viburnum opulus*	0.68	2	g
Hypericum hirsutum	2.72	6	s	*Vicia cracca*	8.84	4	g
Hypericum perforatum	68.71	6	g	*Vicia hirsuta*	21.77	7	g
Hypochoeris radicata	5.44	4	g	*Vicia sativa*	10.2	7	g
Inula conyzae	29.25	5	s	*Vicia sepium*	9.52	2	g
Jacobaea erucifolia	1.36	5	s	*Vincetoxicum hirundinaria*	8.84	2	s
Jacobaea vulgaris	25.17	5	g	*Viola hirta*	88.44	2	s
Juglans regia	0.68	1	g	*Viola riviniana*	4.76	2	g
Knautia arvensis	5.44	4	g				



Appendix 2.4. NMDS ordination on the arcsine transformed plots x species matrix. Restoration time given for every plot.



Appendix 2.5. NMDS ordination on the arcsine transformed plots x emergent groups matrix. Restoration time given for every plot.

Appendix 2.6. Overview of mean trait values for the emergent groups. Emergent group numbers are based on Table 2.2; group 1: Megaphanerophytes, group 2: Forest/shrub species, group 3: Orchids, group 4: Small grassland herbs, group 5: Large herbs & grasses, group 6: Sedges & shallow soil specialists, group 7: Annuals. Descriptions of plant traits given in Appendix 2.2. Trait values given are dependent upon variable type: [1]: mean, [2]:median, [3]:frequency. A.P. = attachment potential, see Appendix 2.2.

emergent group → plant trait ↓	1	2	3	4	5	6	7
plant height[1]	2643	234	273	63	58	37	32
life span[2]	4	4	4	4	4	4	1
rosette type[2]	3	3	2	1	2	2	2
L[2]	5	6	6	7	7	7	7
N[2]	6	5	3	3	5	4	6
flowering start[2]	4	5	5	6	6	5	5
pollen vector: insects[3]	0.36	0.81	0.75	0.83	0.57	0.60	0.10
pollen vector: selfing[3]	0.00	0.13	0.17	0.030	0.19	0.040	0.87
pollen vector: wind[3]	0.64	0.080	0.080	0.12	0.23	0.37	0.030
pollen vector: water[3]	0	0	0	0.010	0	0	0
reproductive type[2]	1.0	1.0	1.0	1.5	2.0	1.5	1.0
diaspore type: fruit[3]	0.79	0.73	0.00	0.65	0.89	0.50	0.45
diaspore type: seed[3]	0.21	0.28	1.00	0.32	0.11	0.46	0.55
diaspore type: spore[3]	0.0	0.0	0.0	0.01	0.0	0.04	0.0
diaspore type: veget.[3]	0.0	0.0	0.0	0.01	0.0	0.0	0.0
seed length[1]	13.99	4.77	0.68	2.34	3.51	1.90	1.68
seed shape[1]	2.27	1.60	2.010	1.65	4.54	1.84	1.65
seed longevity[1]	0.10	0.10	0	0.26	0.35	0.56	0.44
fertility system[2]	3	2	3	3	2	2	1
seed mass[1]	837.90	43.21	0.020	2.23	1.26	0.82	2.44
seed number[1]	3.42	2.24	4.79	2.52	3.41	2.78	2.74
mycorrhizal frequency[2]	3	3	3	3	3	1	2
mycorrhizal type[2]	2	1	3	1	1	1	1
autochory[3]	0.15	0.30	0.15	0.59	0.020	0.76	0.46
hemerochory[3]	0.38	0.080	0.080	0.33	0.57	0.24	0.50
anemochory[3]	0.54	0.080	1.00	0.41	0.78	0.40	0.21
endozoochory[3]	0.38	0.65	0.00	0.81	0.49	0.48	0.79
epizoochory[3]	0.38	0.28	0.080	0.84	0.92	0.72	0.71
dysochory[3]	0.77	0.68	0.00	0.41	0.53	0.44	0.39
nitrogen fixation[3]	0.00	0.050	0.00	0.18	0.00	0.040	0.13
age first flowering[2]	3	2	3	2	1	2	1
leaf size[1]	3110.13	4698.10	2781.13	1546.94	5313.13	789.82	1009.13
A.P. sheep[1]	19.48	44.28	80.93	52.19	75.43	58.44	60.69
A.P. cattle[1]	1.51	5.16	23.19	9.01	13.71	11.45	12.21

Appendix 3.1. Spearman rank correlations of abiotic and spatial variables to restoration age. Test statistic and Spearman *R* given. Spatial autocorrelation analysis for age. Test statistic and Moran's I given. Variables were calculated for each individual grassland patch (n = 43). Significance: *0.05 ≥ *P*-value > 0.01 **0.01 ≥ *P* -value > 0.001 ***0.001 ≥ *P* -value.

	t	R
% bare rock	-1.3	-0.20
% open soil	-1.3	-0.29
soil depth	0.8	0.13
plot inclination	-1.0	-0.16
patch elevation	0.3	0.046
isolation: closest edge	-0.2	-0.035
isolation: closest centroid	-0.4	-0.063
isolation: buffer	1.7	0.26
isolation: Hanski	1.0	0.16
	Z	Moran's I
spatial autocorrelation	0.8	0.03

	AB1	AB2	CO	DLT1	DLT2	FDC	GA	HR	INZ	MB1	MB2	MB3	MV1	MV2	PTB	RI1	RI2	RL1	RL2	RM1	RM2	RT1	RT2	TB	TD	TS1	TS2
AB1	0.000	0.023	0.100	0.071	0.067	0.065	0.059	0.069	0.045	0.069	0.039	0.089	0.051	0.079	0.018	0.064	0.089	0.064	0.073	0.058	0.117	0.050	0.053	0.076	0.052	0.111	0.093
AB2	0.021	0.000	0.091	0.125	0.083	0.109	0.074	0.037	0.051	0.085	0.070	0.115	0.064	0.094	0.079	0.061	0.049	0.051	0.060	0.091	0.129	0.090	0.045	0.030	0.030	0.100	0.131
CO	0.053	0.042	0.000	0.029	0.054	0.065	0.068	0.058	0.059	0.054	0.069	0.043	0.018	0.101	0.071	0.065	0.021	0.039	0.032	0.042	0.090	0.066	0.076	0.094	0.058	0.045	0.036
DLT1	0.041	0.052	0.021	0.000	0.020	0.065	0.034	0.089	0.059	0.033	0.039	0.058	0.019	0.085	0.025	0.051	0.059	0.060	0.042	0.025	0.068	0.043	0.105	0.133	0.075	0.050	0.017
DLT2	0.040	0.038	0.028	0.017	0.000	0.056	0.026	0.046	0.038	0.011	0.013	0.058	0.038	0.089	0.051	0.028	0.030	0.049	0.009	0.023	0.040	0.041	0.090	0.083	0.029	0.043	0.041
FDC	0.039	0.044	0.032	0.033	0.029	0.000	0.089	0.109	0.072	0.105	0.034	0.080	0.078	0.091	0.051	0.090	0.073	0.072	0.071	0.028	0.107	0.029	0.046	0.163	0.052	0.102	0.111
GA	0.031	0.028	0.035	0.027	0.020	0.038	0.000	0.068	0.053	0.033	0.047	0.110	0.029	0.083	0.016	0.038	0.053	0.031	0.054	0.040	0.074	0.024	0.049	0.073	0.055	0.067	0.056
HR	0.042	0.024	0.034	0.043	0.027	0.049	0.032	0.000	0.011	0.061	0.070	0.063	0.021	0.099	0.080	0.036	0.021	0.034	0.014	0.052	0.085	0.077	0.089	0.029	0.043	0.051	0.065
INZ	0.033	0.027	0.034	0.034	0.024	0.038	0.027	0.014	0.000	0.062	0.057	0.069	0.025	0.079	0.045	0.021	0.042	0.033	0.012	0.039	0.090	0.048	0.068	0.054	0.040	0.032	0.066
MB1	0.042	0.041	0.032	0.025	0.015	0.046	0.022	0.035	0.036	0.000	0.048	0.029	0.026	0.084	0.043	0.046	0.056	0.031	0.014	0.039	0.016	0.052	0.097	0.064	0.065	0.034	0.026
MB2	0.032	0.034	0.035	0.027	0.016	0.023	0.027	0.037	0.032	0.028	0.000	0.036	0.048	0.091	0.036	0.074	0.054	0.070	0.034	0.025	0.061	0.039	0.062	0.105	0.032	0.064	0.082
MB3	0.051	0.052	0.026	0.030	0.028	0.038	0.047	0.039	0.038	0.023	0.025	0.000	0.010	0.109	0.071	0.111	0.078	0.043	0.031	0.036	0.036	0.079	0.108	0.118	0.109	0.034	0.036
MV1	0.036	0.034	0.022	0.019	0.025	0.037	0.025	0.022	0.024	0.025	0.032	0.022	0.000	0.054	0.020	0.052	0.036	-0.005	0.025	0.002	0.041	0.045	0.060	0.061	0.067	0.013	0.001
MV2	0.041	0.040	0.044	0.037	0.038	0.037	0.035	0.044	0.037	0.039	0.039	0.047	0.031	0.000	0.062	0.075	0.058	0.065	0.087	0.047	0.096	0.061	0.081	0.099	0.083	0.080	0.088
PTB	0.023	0.036	0.038	0.023	0.029	0.028	0.018	0.042	0.029	0.028	0.026	0.038	0.023	0.028	0.000	0.037	0.074	0.045	0.060	0.017	0.069	0.013	0.040	0.105	0.060	0.057	0.034
RI1	0.042	0.033	0.034	0.030	0.021	0.040	0.022	0.027	0.020	0.030	0.036	0.048	0.033	0.032	0.024	0.000	0.028	0.038	0.021	0.054	0.073	0.065	0.084	0.074	0.026	0.044	0.056
RI2	0.052	0.032	0.017	0.030	0.023	0.034	0.032	0.023	0.030	0.034	0.032	0.037	0.028	0.032	0.040	0.024	0.000	0.053	0.028	0.049	0.095	0.057	0.059	0.041	0.019	0.052	0.040
RL1	0.031	0.023	0.028	0.031	0.026	0.035	0.017	0.023	0.021	0.024	0.034	0.030	0.015	0.030	0.025	0.024	0.032	0.000	0.023	0.017	0.044	0.053	0.038	0.052	0.048	0.020	0.060
RL2	0.043	0.032	0.022	0.024	0.012	0.034	0.028	0.018	0.018	0.017	0.023	0.021	0.022	0.038	0.032	0.020	0.022	0.020	0.000	0.024	0.019	0.055	0.077	0.084	0.031	0.023	0.053
RM1	0.037	0.042	0.031	0.021	0.021	0.025	0.028	0.032	0.029	0.029	0.025	0.029	0.015	0.027	0.021	0.031	0.032	0.020	0.022	0.000	0.031	0.014	0.048	0.098	0.048	0.031	0.037
RM2	0.058	0.055	0.049	0.036	0.025	0.050	0.036	0.043	0.045	0.021	0.036	0.031	0.031	0.042	0.036	0.038	0.051	0.028	0.022	0.024	0.000	0.093	0.122	0.136	0.115	0.028	0.051
RT1	0.039	0.042	0.035	0.033	0.029	0.025	0.022	0.041	0.031	0.032	0.028	0.040	0.035	0.033	0.021	0.032	0.033	0.030	0.032	0.027	0.049	0.000	0.015	0.111	0.054	0.078	0.062
RT2	0.037	0.027	0.033	0.045	0.039	0.026	0.027	0.039	0.032	0.043	0.032	0.046	0.035	0.036	0.026	0.034	0.029	0.027	0.036	0.033	0.058	0.016	0.000	0.101	0.039	0.094	0.111
TB	0.034	0.017	0.046	0.056	0.040	0.063	0.030	0.022	0.030	0.035	0.045	0.054	0.034	0.043	0.045	0.040	0.033	0.024	0.039	0.044	0.053	0.051	0.045	0.000	0.063	0.081	0.101
TD	0.037	0.025	0.032	0.033	0.022	0.029	0.031	0.030	0.030	0.037	0.025	0.050	0.034	0.037	0.033	0.026	0.022	0.030	0.025	0.029	0.054	0.037	0.029	0.035	0.000	0.087	0.104
TS1	0.057	0.046	0.028	0.026	0.022	0.044	0.032	0.031	0.025	0.024	0.032	0.023	0.021	0.036	0.031	0.023	0.030	0.021	0.017	0.025	0.024	0.037	0.041	0.043	0.042	0.000	0.038
TS2	0.051	0.057	0.023	0.017	0.024	0.046	0.034	0.037	0.036	0.023	0.040	0.025	0.017	0.040	0.027	0.033	0.026	0.035	0.028	0.026	0.034	0.038	0.046	0.050	0.046	0.025	0.000

Appendix 4.1. Pairwise genetic differentiation among *Origanum vulgare* populations. Lower left triange: F_{ST} estimates; Upper right triange: G'_{ST} estimates. Significant values are shaded in grey.

Appendix 4.2. Pairwise genetic differentiation among *Origanum vulgare* populations.
Lower left triangle: Jost's D estimates Significant values are shaded in grey.

	AB1	AB2	CO	DLT1	DLT2	FDC	GA	HR	INZ	MB1	MB2	MB3	MV1	MV2	PTB	RI1	RI2	RL1	RL2	RM1	RM2	RT1	RT2	TB	TD	TS1	TS2
AB1	0																										
AB2	0.015	0																									
CO	0.07	0.063	0																								
DLT1	0.048	0.085	0.02	0																							
DLT2	0.044	0.055	0.037	0.014	0																						
FDC	0.043	0.073	0.045	0.044	0.037	0																					
GA	0.039	0.049	0.048	0.023	0.017	0.06	0																				
HR	0.043	0.023	0.038	0.058	0.029	0.07	0.043	0																			
INZ	0.029	0.033	0.04	0.039	0.024	0.048	0.035	0.007	0																		
MB1	0.046	0.057	0.038	0.022	0.007	0.072	0.022	0.039	0.041	0																	
MB2	0.026	0.048	0.049	0.027	0.009	0.023	0.032	0.045	0.038	0.033	0																
MB3	0.061	0.08	0.031	0.041	0.04	0.056	0.077	0.041	0.047	0.02	0.025	0															
MV1	0.034	0.042	0.012	0.013	0.025	0.052	0.019	0.013	0.016	0.017	0.033	0.007	0														
MV2	0.053	0.063	0.071	0.058	0.06	0.061	0.056	0.064	0.052	0.057	0.063	0.076	0.036	0													
PTB	0.011	0.052	0.049	0.016	0.033	0.034	0.01	0.05	0.029	0.029	0.024	0.048	0.013	0.041	0												
RI1	0.041	0.038	0.044	0.033	0.018	0.058	0.024	0.022	0.013	0.03	0.049	0.075	0.034	0.048	0.024	0											
RI2	0.06	0.033	0.015	0.04	0.02	0.05	0.036	0.013	0.028	0.038	0.037	0.055	0.024	0.04	0.05	0.018	0										
RL1	0.042	0.033	0.027	0.04	0.032	0.048	0.02	0.021	0.021	0.02	0.048	0.029		0.043	0.03	0.024	0.036	0									
RL2	0.048	0.039	0.022	0.028	0.006	0.048	0.036	0.009	0.008	0.01	0.023	0.021	0.016	0.059	0.04	0.013	0.019	0.015	0								
RM1	0.038	0.06	0.029	0.016	0.015	0.019	0.027	0.033	0.025	0.026	0.017	0.025	0.001	0.031	0.011	0.034	0.033	0.011	0.016	0							
RM2	0.077	0.085	0.062	0.045	0.026	0.07	0.048	0.052	0.058	0.011	0.041	0.024	0.027	0.063	0.044	0.046	0.063	0.028	0.012	0.02	0						
RT1	0.034	0.061	0.046	0.029	0.027	0.019	0.016	0.05	0.032	0.035	0.027	0.056	0.031	0.041	0.009	0.043	0.039	0.035	0.038	0.009	0.062	0					
RT2	0.036	0.03	0.054	0.074	0.062	0.031	0.034	0.058	0.046	0.068	0.044	0.078	0.042	0.056	0.027	0.056	0.041	0.026	0.053	0.033	0.083	0.01	0				
TB	0.048	0.019	0.063	0.089	0.054	0.109	0.048	0.017	0.034	0.042	0.071	0.08	0.04	0.065	0.068	0.046	0.027	0.033	0.055	0.064	0.087	0.074	0.068	0			
TD	0.034	0.019	0.04	0.05	0.019	0.034	0.036	0.027	0.026	0.043	0.022	0.076	0.045	0.056	0.04	0.017	0.013	0.031	0.02	0.032	0.075	0.036	0.026	0.04	0		
TS1	0.073	0.066	0.031	0.033	0.028	0.068	0.044	0.031	0.021	0.022	0.044	0.023	0.008	0.053	0.037	0.028	0.035	0.013	0.015	0.02	0.018	0.053	0.064	0.052	0.058	0	
TS2	0.062	0.089	0.025	0.011	0.027	0.076	0.037	0.041	0.044	0.018	0.057	0.024	0.001	0.06	0.022	0.036	0.027	0.04	0.036	0.024	0.033	0.042	0.078	0.067	0.07	0.025	0

Appendix 5.1. Overview of the observed species in the seed bank. Presence of each species is indicated for the seed bank of old, middle-aged (m.a.) and young grasslands patches separately.

species	old	m.a.	young	species	old	m.a.	young
Achillea millefolium		x		*Kickxia elatine*		x	
Agrostis capillaris	x	x	x	*Knautia arvensis*	x		
Agrostis stolonifera	x	x	x	*Koeleria macrantha*	x	x	
Anagallis arvensis		x		*Lapsana communis*		x	x
Aphanes arvensis			x	*Leontodon hispidus*	x		x
Arabis hirsuta		x		*Leucanthemum vulgare*	x	x	x
Arenaria serpyllifolia	x	x	x	*Linaria vulgaris*		x	
Arrhenatherum elatius	x		x	*Lotus corniculatus*	x	x	x
Atropa belladonna	x	x		*Luzula campestris*	x	x	x
Barbarea stricta		x		*Medicago lupulina*	x	x	x
Betula pendula	x	x	x	*Melica ciliata*	x	x	
Brachypodium pinnatum	x	x	x	*Melica nutans*	x	x	x
Calluna vulgaris	x	x	x	*Mercurialis annua*	x	x	x
Campanula rapunculus	x	x		*Moehringia trinervia*	x		x
Campanula rotundifolia	x	x	x	*Myosotis arvensis*		x	x
Cardamine hirsuta	x	x	x	*Myosoton aquaticum*		x	
Carduus crispus	x	x	x	*Origanum vulgare*	x	x	
Carex flacca	x	x	x	*Papaver rhoeas*	x	x	x
Carex remota	x	x		*Picris hieracioides*	x	x	x
Carex sp.		x		*Pimpinella saxifraga*	x		
Centaurea jacea	x	x	x	*Plantago lanceolata*	x	x	x
Centaurium erythraea	x	x	x	*Plantago major*	x	x	x
Cerastium fontanum	x	x	x	*Poa angustifolia*	x	x	x
Cerastium pumilum		x		*Poa annua*		x	x
Chaenorhinum minus		x	x	*Poa compressa*		x	
Cirsium arvense	x	x	x	*Poa pratensis*	x	x	x
Cirsium palustre		x	x	*Poa sp*	x	x	x
Cirsium vulgare		x	x	*Polygala sp.*	x		x
Clematis vitalba		x		*Potentilla neumanniana*	x	x	x
Clinopodium acinos		x	x	*Primula veris*			x
Conyza canadensis		x	x	*Prunella vulgaris*	x	x	x
Dactylis glomerata		x	x	*Ranunculus bulbosus*		x	
Danthonia decumbens	x	x		*Ranunculus repens*	x	x	x
Daucus carota		x	x	*Reseda luteola*	x	x	
Digitalis lutea		x		*Rorippa sylvestris*			x
Echium vulgare	x			*Rubus fruticosus*		x	x

Epilobium ciliatum			x	*Rubus idaeus*		x	x
Epilobium hirsutum		x	x	*Rumex obtusifolius*		x	x
Epilobium lanceolatum			x	*Sambucus nigra*	x		
Epilobium montanum		x		*Sanguisorba minor*	x	x	x
Epilobium sp.		x	x	*Scabiosa columbaria*	x	x	x
Epilobium tetragonum		x	x	*Scrophularia sp*	x		
Erica tetralix	x			*Sedum acre*		x	
Eupatorium cannabinum	x	x	x	*Sedum album*		x	x
Euphorbia helioscopia		x	x	*Sinapis alba*			x
Fallopia convolvulus		x		*Sisymbrium officinale*	x		
Festuca lemanii	x	x		*Solanum dulcamara*		x	x
Fragaria vesca	x	x	x	*Sonchus asper*	x	x	x
Fumaria officinalis		x		*Sonchus oleraceus*		x	x
Galium aparine			x	*Stachys annua*	x	x	
Galium pumilum	x	x	x	*Stachys officinalis*		x	
Galium verum	x	x		*Taraxacum officinale*	x	x	x
Genista sagittalis	x			*Teucrium chamaedrys*	x	x	
Genista tinctoria	x	x		*Thymus praecox*	x	x	
Geranium columbinum			x	*Thymus pulegioides*	x	x	
Geranium molle		x		*Torilis japonica*		x	
Geranium robertianum			x	*Trifolium campestre*	x	x	x
Geum urbanum	x	x	x	*Trifolium hybridum*		x	
Globularia punctata	x			*Trifolium medium*	x		
Helianthemum nummularium	x	x	x	*Trifolium repens*		x	
Hieracium pilosella	x	x		*Urtica dioica*	x	x	x
Hippocrepis comosa			x	*Verbascum thapsus*		x	x
Holcus lanatus	x	x	x	*Veronica arvensis*	x	x	x
Holcus mollis		x		*Veronica officinalis*		x	x
Hypericum hirsutum		x		*Veronica serpyllifolia*		x	x
Hypericum humifusum	x	x	x	*Vicia cracca*	x		x
Hypericum perforatum	x	x	x	*Vicia hirsuta*		x	x
Inula conyza	x	x	x	*Vicia tetrasperma*		x	
Jacobaea vulgaris	x	x	x	*Viola hirta*	x	x	x
Juncus effusus	x			Unknown sp.		x	

Appendix 5.2. Parameter estimates of performed SumF analysis relating trait (CWM) composition to grassland patch age (*F*-ratio) based on 9999 randomisations. Results are also given for the bootstrap LMM relating individual traits to restoration age (mean difference (contrasts)) based on 9999 bootstraps. Traits are sorted by decreasing *F*-ratio, with significant effects in italics. Explanation of the different traits can be found in Appendix 2.2. m.a.: middle-aged (age group). Significance: *0.05 ≥ *P*-value > 0.01 **0.01 ≥ *P*-value > 0.001 ***0.001 ≥ *P*-value.

Trait	F-ratio	contrast old – m.a.	contrast m.a. – young	contrast old – young
Ellenberg N	40.45	-0.185***	-0.077*	-0.253***
hemerochory	19.78	-0.146***	-0.093*	-0.252***
autochory	16.36	0.143***	0.083*	0.238***
life span	13.92	0.106***	0.060	0.196***
life form: therophyte	13.11	-0.106***	-0.034	-0.161***
leaf size	10.24	-0.032***	-0.013	-0.046**
fertility system	9.58	0.084***	-0.010	0.067**
dysochory	9.19	-0.014	-0.139***	-0.153***
seed shape	8.69	-0.038***	-0.007	-0.046**
life form: chamaephyte	8.40	0.072**	0.050*	0.109***
seed longevity	8.18	-0.076**	0.029	-0.058**
life form: phanerophyte	6.06	-0.023*	-0.026	-0.052*
seed mass	5.76	0.058***	-0.062***	0.007
seed number	5.61	-0.055**	0.0002	-0.058**
pollen vector: insects	4.89	0.076*	0.038	0.126**
pollen vector: selfing	4.81	-0.074*	-0.036	-0.125**
rosette type	4.62	0.015	0.077*	0.082**
Ellenberg L	4.40	0.024*	0.016	0.035*
epizoochory	3.76	0.068**	-0.019	0.050
flowering start	3.41	0.013	0.022	0.040**
age first flowering	3.36	0.060*	0.007	0.069
seed length	3.36	-0.009	-0.026*	-0.025**
plant height	2.48	0.022	0.013	0.043*
reproductive type	1.91	-0.047*	0.021	-0.019
life form: hemicryptophyte	1.61	0.043	0.032	0.072
endozoochory	1.59	0.053	-0.040	-0.031
mycorrhizal type: ericoid	1.23	0.015	0.012	0.026
mycorrhizal frequency	0.86	0.039	-0.001	0.040
mycorrhizal type: arbuscular	0.49	0.011	-0.031	-0.020
mycorrhizal type: ecto	0.46	-0.003	-0.002	-0.004
life form: geophyte	0.22	0.015	-0.015	-0.002
anemochory	0.09	0.014	-0.004	0.012
pollen vector: wind	0.06	-0.0005	-0.003	0.0009
nitrogen fixation	0.01	0.001	-0.002	-0.008

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